Saskatchewan
Agriculture
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# ADF

## AGRICULTURE DEVELOPMENT FUND

## *FINAL REPORT*

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**DEVELOPMENT OF SYNTHETIC EXTENDERS FOR CRYOPRESERVATION OF BULL AND BOAR SEMEN**

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**Abstract/Summary**

Preliminary studies at the University of Saskatchewan demonstrated that it was possible to replace egg yolk with phosphotidylcholine of soybean origin in a bovine semen extender for cryopreservation, and that high pressure homogenization was most efficacious in emulsifying the lipid preparations. Studies at Boviteq identified the oil-phospholipid (1:1.5) emulsification in water that maintained viability, motility and acrosome integrity of bovine semen. The synthetic extender conserved sperm function numerically better than control egg-yolk extender. The synthetic formulation also had a shelf life of more than 3 months, and the average particle size was 189.4 nm remaining unchanged through an 8 month period.

Regardless of the time of incubation, all bulls had a lower percentage of dead spermatozoa after preservation in the 0 month or 3-month old synthetic extender when compared to freshly prepared egg yolk extender. In vitro data showed that the synthetic extender was superior to the egg yolk extender in many aspects of sperm function, even after 8 months of storage. This was also true for the autoclaved version although the advantage began to decrease after 3 months.

Various additives were analyzed; all parameters assessed indicated no benefit to the addition of hyaluronan to the synthetic extender. The addition of an antioxidant improved viability, intracytoplasmic calcium levels, progressive motility and sperm velocity parameters. These data indicate that antioxidants could have a positive effect on the fertility of semen cryopreserved in the synthetic extender.

Artificial insemination of superstimulated cows with semen cryopreserved in the synthetic extender resulted in fertilization rates comparable to that of semen frozen in control egg-yolk extender. Following artificial inseminations of dairy cows in a controlled field study, no statistical difference in fertility was seen between semen cryopreserved in the synthetic extender or the egg yolk extender. However data are still being collected.

In the study of a swine synthetic extender, liposomes were prepared in Beltsville (BTS) extender from two extracts of soy lipids, one primarily phosphatidylcholine ($Soy_{PC}$), the other enriched in phosphatidylethanolamine ($Soy_{PE}$) and compared to liposomes made from selected phospholipids from defined sources. Dynamic light scattering and electron microscopy revealed small liposomes of a consistent size that remained consistent after refrigeration for up to 72 h. Flow cytometry and fluorescence microscopy showed that the liposomes fused to sperm membranes.

Boar sperm were stored in various concentrations of liposomes for 1, 7 or 10 days and tested for viability and motility. Liposomes prepared from $Soy_{PC}$ or combined $Soy_{PC}$ / $Soy_{PE}$ increased viability during 18°C storage; however, sperm did not survive cryopreservation in liposomes. Acrosomal status clearly indicated that liposomes containing $Soy_{PE}$ or low concentrations of $Soy_{PC}$ either promoted spontaneous acrosome reactions or prevented normal capacitation in stored sperm. $Soy_{PC}$ liposomes at 1.5 mg/mL in BTS were selected for field testing; sows were inseminated after 0 or 7 days storage and compared to fresh BTS. Pregnancy results are still being collected, but sows inseminated at both 0 and 7 days have been successfully impregnated.

In summary, the bovine synthetic extender based on $Soy_{PC}$ plus canola oil was more efficacious that a commercial egg yolk extender in the cryopreservation of bovine semen, and at least comparable in a field study currently being undertaken. Our industry partners are pleased with its potential. The porcine synthetic extender based on 1.5 mg/mL $Soy_{PC}$ would appear to be effective over 7 days; however, some additional studies are required. Cryopreservation with the synthetic extender is still elusive and still requires a great deal more research.

**Background**

This project was originally funded at slightly less than a 50% level by the Saskatchewan Agriculture Development Fund (ADF) with the instructions that matching funds be procured from industry. We sought and received $30,000 (over the 3 years) from Sask Pork. At the same time, a commercial partner, Bioniche Animal Health, Belleville, Ontario, that wanted to have marketing rights on the product was located. They agreed to support the project at a level of $30,000 per year (total of $90,000). In the meantime, Palasz (the Principal Investigator) accepted a position with Alliance Boviteq Inc. Ste. Hyacinthe, Quebec, who also had a strong interest in the project; they agreed to support it financially and get involved in conducting research.

Bionche Animal Health and Alliance Boviteq Inc. were both supportive of their respective roles in the project, but patent issues on sperm membrane lipids held by Dr. Mary Buhr, University of Guelph became an issue. Dr. Buhr also had expertise on pig semen so she was invited to become a collaborator in the project. It took a great deal of time to sort out rights and responsibilities and whether an agreement should be between the two commercial partners, or whether each should negotiate an agreement with the University of Saskatchewan. We finally arrived at a comprehensive research agreement which involved all four partners. A Research Collaboration Agreement, back-dated to May 1, 2004 among all participants was signed in March, 2005. Boviteq and the University of Guelph also sought additional funding, which was obtained. As a result, four sources of funding, two universities and two commercial partners became involved with this project. The involvement of these industrial partners also meant that research priorities were directed primarily toward the development of a commercial product(s).

In the meantime, Palasz resigned as Principal Investigator and took employment in Spain. Mapletoft assumed the overall supervision of the project with responsibilities for budgets, ensuring scientific scrutiny, progress reports etc. Research activities were transferred to Alliance Boviteq Inc. in Quebec (bovine) and the University of Guelph (swine). However, the four-way agreement stipulated that a Research Committee and Joint Steering Committee representing the four partners be set up to oversee research activities and approve management and expenses, respectively. Upon the approval of research progress reports by the Research Committee and expenses by the Joint Steering Committee, expenses incurred in Quebec and Guelph were to be invoiced to the University of Saskatchewan (and other funding agencies) for payment.

This Final Report will be divided into two parts; research conducted at the University of Saskatchewan (July 2002 to May 2003) and the bovine semen research conducted at Boviteq (July 2004 to February 2007) and swine semen research conducted at the University of Guelph (November 2004 to February 2007). The studies at the University of Saskatchewan conducted over the first year must be viewed as preliminary background information which provided the foundation for the subsequent studies, while the more recent studies at Boviteq (bovine) and University of Guelph (swine) must be viewed as most definitive, and product-oriented.

## Introduction

The Canadian beef and dairy industries are the second and fourth largest sectors of the Canadian agri-food economy. Today, the cattle industry worldwide depends on the use of artificial insemination (AI) and frozen semen for the continual management and improvement of the high valued genetics of their herds. Extensive use of AI has permitted an accelerated gain in genetic improvement in the dairy industry, and has the potential to do the same in the beef industry. Semen cryopreservation is possible by freezing sperm cells in cryoprotectants that contain lipoproteins such as egg yolk; in either case, animal proteins from outside sources are present in the frozen semen.

As for the swine industry, the use of frozen-thawed boar semen is becoming more popular for commercial breeding by AI. However, fertility is not very high with frozen swine semen; liquid semen AI is more widely used due to its lower cost and higher fertility. Both of these techniques necessitate the dilution of swine semen in an extender that contains some form of animal protein such as bovine serum albumin (BSA).

In response to recent concern of the potential transfer of infectious diseases across borders, the primary objective of this project is to develop a fully synthetic semen extender that incorporates plant phospholipids such as soybean extracts as a replacement for the animal products used today. Very few commercial products exist today that can accomplish this and maintain acceptable fertility in the field. In addition, new knowledge on the make-up and function of sperm membranes and the possibility of protecting sperm membranes with exogenous phospholipids, fatty acids, sterols, glycosaminoglycans and antioxidants leads us to believe that cryoprotective solutions can be improved and sperm survival rates can be improved.

Progress in semen cryopreservation has reached a plateau and with present techniques, post-thaw sperm survival remains 40 - 50% of sperm population in bull, and less than 25% in the boar. Factors that affect the survival of frozen-thawed sperm include composition of the sperm membranes (Park and Lynch, 1992; Douard et al., 2000; He et al., 2001), cooling and warming rates, choice and concentration of the cryoprotectant (Fiser and Fairfull, 1990), type and size of the freezing container (Bamba and Cran, 1988) and chemical and physical characteristics of the freezing medium (Hirai et al., 1997; Hallak et al., 2000; Palasz et al., 2000). Cryoprotectants such as glycerol or ethylene glycol must be present in 1.0 – 2.0 M concentrations and to be effective, these cryoprotectants must permeate sperm membranes (Mazur, 1984). However, attempts to cryopreserved sperm in glycerol without the addition of egg yolk has been highly inefficient (Hallak et al., 2000), and when BSA was substituted for egg yolk, post-thaw semen viability was very low. Therefore, egg yolk and milk are currently used in extenders for the cryopreservation of human and domestic animal semen (Sugino et al., 2000). However, despite improvements in collection and processing, including filtration and gamma radiation, all components of animal origin are potentially contaminated with infectious agents (Rossi et al., 1990).

Understanding sperm membranes dynamics and specific lipid composition, distribution in outer and inner sperm membranes, lipid-proteins and lipid-lipid interactions at both physiological and low temperatures are essential to develop efficient extenders for the cryopreservation of sperm from all species (Buhr et al., 1991). Spermatozoa apparently metabolize endogenous phospholipids during in vitro storage (Scoot and Dawson, 1968), and a significant decrease in phosphatidylcholine, which is a major phospholipid of chicken, but not other avian species egg yolk, has been observed during cooling of turkey spermatozoa (Douard et al., 2000). At the same

time, it was demonstrated that mammalian membranes can readily modify and take up exogenous lipids from the culture medium (Spector and Yorek, 1985). Recent findings suggest that cell membrane composition can be manipulated and thermobehavior can be changed (Buhr et al., 1991). The major phospholipid of mitochondria and sperm head and nuclear membrane bilayers is phosphatidylcholine (Parks and Lynch, 1992), which is present in high concentrations in egg yolk. It has also been shown to be a potent antioxidant (Sugino et al., 2000). In addition, catechin of plant origin (Lee et al., 2000) has been shown to be a very effective inhibitor of lipid peroxidation of the phospholipid bilayer (Bilodeau et al., 2000) and when combined with phosphatidylcholine of plant origin was an excellent substitute for egg yolk for the cryopreservation of bull spermatozoa (Palasz, unpublished). We hypothesized that careful selection of a phospholipid mixture of plant origin could be substituted for products of animal origin in extenders for boar and bull semen, reducing potential for the transmission of infectious disease, and at the same time improving the ability of spermatozoa to survive cold storage.

A series of experiments were designed to define the role of exogenous phospholipids, fatty acids, sterols, glycosaminoglycans and antioxidants in the successful cold storage and cryopreservation of bull and boar spermatozoa. The following specific objectives were proposed: 1) To determine the effect of exogenous phospholipids of plant origin and/or synthetic sterols on semen chilling sensitivity and freezing, 2) To determine the effect of chemical nature of exogenous phospholipids on sperm survival, 3) To determine the effect of the antioxidant properties of catechins of plant origin and others antioxidants such as hyaluronan, against sperm membrane lipid peroxidation during cooling, 4) To determine the effect of cooling/warming rate, type and concentration of cryoprotectant and osmolality of semen extender on sperm survival, 5) To determine the effect of timing of the addition of antibiotics to the semen extender on antibiotic effectiveness and post-thaw semen viability, 6) To produce commercially-prepared semen extenders with phospholipids of plant origin for cryopreservation of bull and boar spermatozoa, with extended shelf life, appropriate packing etc., 7) To field test the extenders which produce the best results in in-vitro studies and in artificial insemination programs.

## PART A - UNIVERSITY OF SASKATCHEWAN (JULY 2002 – MAY 2003)

In our previous, ADF-funded studies, we showed that egg yolk, an essential component of bull semen extenders, can be replaced by phosphatidylcholine of soybean origin (Palasz et al, 2002). However, the originally prepared extender was cloudy and a method of solubilization of phosphatidylcholine had to be developed to make it translucent and easier to work with. Improvement of physical properties of the extender, making it commercially acceptable was our first priority. In the first series of experiments, the objective was to make the existing phosphatidylcholine extender translucent without sacrificing efficacy. Specifically, we wanted to determine the effects of different solubilization procedures, and the use of synthetic water-soluble surface-active agents, on the size and distribution of the liposomes and the viability of the bull semen during cooling and after freeze-thawing. The following methods of solubilization were investigated: 1) heating to different temperatures and stirring, 2) heating and filtration through 0.22um filter, 3) sonication only (at $22^0$C), and 4) sonication with the addition of surfactants, and 4) high-pressure homogenization.

**Rationale:** Phospholipids in a low lipid/water ratio naturally produce bilayer structures, similar to biological membranes. However, when traditional stirring methods were used to solubilize lipids, large sized multilamellar vesicles are formed. We hypothesized that improved solubilization of the lipid preparation would produce smaller sized vesicles that would be transparent and easier to use while maintaining efficacy. Sonication is a convenient way to produce small unilamellar vesicles. Disruption of large sized multilamellar vesicle suspensions using sonic energy (sonication) typically produces small unilamellar vesicles with diameters in the range of 15 to 50 nm. This increases active surface area of the liposomes making them more effective. Whether sonification has an effect on sperm viability during cryopreservation must be established. Other means of reducing the size of lipid vesicles included the use of surface-active agents. There is evidence that the addition of surfactants to egg yolk diluents improves the post-thaw motility, acrosomal integrity, survival and fertility of sperm from several species, including wildlife species. The benefits can be explained by chemical properties of surfactants that modify egg yolk phospholipids, and make them readily available or more efficient in the interactions with sperm membranes. The size of the lipid vesicles is the second most important factor in the semen extender, behind phospholipid-fatty acid composition.

### General Materials and Methods

Semen was collected from beef bulls twice weekly. After preliminary semen evaluation, aliquots of semen (>70% motile) from each ejaculate were diluted at $37^0$C with each of the extender preparations tested to a concentration of $120 \times 10^6$ spermatozoa per mL. Diluted semen was cooled to $4^0$C over 4 h, and then frozen in 0.25 mL straws in liquid nitrogen vapor (10 cm above the surface for 10 min) and then plunged into liquid nitrogen (unless other specific cooling/freezing protocols were specified). The effects of various treatments on the sperm motility were evaluated under the microscope after each stage of the cooling/freezing procedure. Parameters of motility of the raw semen prior to processing were assessed and used as a reference. Frozen semen was thawed at $37^0$C in a water bath for 20 sec. The same experiments were replicated by cooling semen to 0°C (ice water); results were essentially the same.

Observation of individual motility and estimation of the percentage of progressively motile cells provides information about sperm viability and membrane integrity, as well as the morphological integrity of spermatozoa. Motile spermatozoa are dependent upon pH, temperature and osmolarity. For this reason, new, perfectly clean, warm slides and cover slips were used.

Individual directional motility, mass motility (swirls and eddies) and quality of sperm movement were determined under a phase-contrast microscopy at 100x magnification. Sperm Morphology was evaluated by phase-contrast microscopy at 1000x magnification after fixation of semen sample in a 2% gluteraldehyde solution. Sperm viability was quantitated microscopically by counting live sperm stained with the eosin-nigrosin stain. Acrosome integrity was evaluated by phase-contrast microscopy at 1000x magnification.

In the following series of experiments, two concentrations of phosphatidylcholine preparations (7.3 and 3.65%) were tested. Although initial results indicated that a concentration of 3.65% phosphatidylcholine produced slightly lower sperm motility after freezing than the 7.3% preparation, we decided to test both preparations further in order to improve the use of the preparation with the lower lipid concentration because of significantly reduced number of large size vesicles. Regular commercial extenders (Triladyl which requires egg yolk, and Andromed which contained soy-based phospholipids) were used as controls. Each experiment was replicated at least three times.

In **Experiment 1**, bull semen extenders containing two different concentrations of phosphatidylcholine (7.3 and 3.65 %) were compared with Triladyl extender containing egg yolk and Andromed extender containing no egg yolk. The post thaw motility of semen frozen in the extender containing 7.3% phosphatidylcholine was as high if not higher (45%) than in two control extenders (Triladyl with egg yolk and Andromed without egg yolk both 35%) which were not different from the extender containing 3.65% phosphatidylcholine (35%).

In **Experiment 2**, the effect of temperature on solubilization of phosphatidylcholine, the size of lipid vesicles in the extender and post-thaw semen motility was examined. There was a visible effect of temperature; the optimum solubilization temperature was $60^0$C. Heating improved solubilization, (as judged by a lower number of large vesicles visible under microscope), but efficacy seemed to decrease as high temperatures were achieved. At both concentrations of phosphatidylcholine, post-thaw viability at 25, 60 and $70^0$C did not differ from the egg yolk extender. However, solubilization at 80, 90 and $100^0$C resulted in significantly lower post-thaw viabililty ($P<0.05$).

In **Experiment 3**, the combined effect of solubilization temperature and filtration of the lipid preparations on the size of lipid vesicles in the extender and post-thaw semen motility were examined. There was no additional benefit of filtration of lipids dissolved in a water solution at different temperatures on the post thaw semen motility. However, there was an improvement in the transparency of the extender. The optimum temperature for solubilization was $60^0$C.

In **Experiment 4**, the addition of different surfactants to the phosphatidylcholine preparations to reduce the number of large vesicles while maintaining post-thaw semen viability was examined. The addition of Tween 20, Orvus ES paste or Pluronic F-68 seemed to improve lipid solubilization and extender transparency, but there was a negative effect of all surfactants on post-thaw semen viability.

In **Experiment 5**, the effect of sonication on the size of phosphatidylcholine lipid unilamellar vesicles and post-thaw bull semen motility was examined. Sonication of the whole extender, including glycerol, was compared to heating and stirring. Sonication (as judged by light microscopy) significantly reduced the number of large unilamellar vesicles and greatly improved the transparency of the extender, but sperm viability and motility were reduced. .

In **Experiment 6**, the effect of pH and osmolarity on post-thaw semen viability, and the stability of the pH and osmolarity during extended storage were examined. There was a negative effect of

extender pH below 6.7 on the post thaw semen motility in both concentrations of phosphatidylcholine, but it was less profound in the 7.3% preparation. Sperm motility was equal to that of Triladyl and egg yolk at the higher pH (6.8 to 7.0). High osmolarity values were clearly detrimental to sperm survival; sperm survival was satisfactory when osmolarity was maintained close to that of Triladyl (1065 – 1430 mmol/kg).

In **Experiment 7**, the effect of the addition of pyruvate, as an energy source, to the phosphatidylcholine semen extender on post-thaw viability of bull semen was examined. Although the addition of exogenous pyruvate to both concentrations of phosphatidylcholine slightly improved post-thaw sperm motility, differences were not significant.

**Experiment 8** was designed to compare concentration of phosphatidylcholine and sonication or heating/filtration on the efficacy of the extender. We previously demonstrated that heating improved solubility of the phospholipid, but efficacy seemed to decrease with increasing temperature. There was no additional effect of filtration of lipids dissolved in water at different temperatures on post-thaw semen motility. However, there was an improvement in the transparency of the extender. The optimum temperature for solubilization was $60^0$C.

Sonicated or heated/filtered extenders were prepared as described above. Semen was prepared in the respective extenders or Triladyl plus egg yolk or AndroMed extenders in three replicates, frozen, thawed and examined as described. Although sperm viability did not differ between sonicated or heated/filtered phosphatidylcholine extenders and Triladyl or AndroMed, the techniques used to solubilize the phosphatidylcholine extenders were not highly repeatable. Although the sonicated solutions were initially clear, they had precipitated out within 2 weeks. High speed mixing was also attempted, but as very small vesicles were still visible this method was discontinued. It was decided to pursue high pressure homogenization as an alternative.

**High-pressure homogenisers** are used for liposome research and production. A whole range of dispersions to reduce particle size as well as make the size distribution more uniform can be achieved through high-pressure homogenisers. Homogenisers are involved in the processing of dispersions such as drugs, herbal extracts and lipids. Only homogenisers offer high-pressure homogenization or high-pressure membrane filtration or the combined benefits of homogenization and filtration processes. We hypothesized that the use of high-pressure homogenization of phosphatidylcholine lipid preparations would allow for a further reduction in liposome size, increasing the active surface area of the liposomes and making them more accessible to the sperm. The reduced size and greater dispersion of the lipid particles should also improve clarity and transparency of the extender. Therefore, subsequent studies were designed to take advantage of high-pressure homogenization of phosphatidylcholine preparations.

**Experiment 9** was designed to compare high-pressure homogenization with sonication with or without heating/filtration of the phosphatidylcholine-based extenders. There was no significant difference among extenders in sperm survival; the sonicated and heated/filtered extenders continued to have limitations. Indeed, following sonication, the phosphatidylcholine preparation was clear, but it became cloudy again within 2 weeks, while samples prepared by high pressure homogenization were still transparent after 4 weeks. In addition, sonication produced a great deal of heat in the preparation (it would also appear that temperature must be controlled with high-pressure homogenization, but this is standard procedure), and when temperature was not controlled, cryoprotective efficacy was shown to decrease. The survival rate of sperm in the homogenized 7.5% phosphatidylcholine extender encouraged our pursuit of this technology.

**Experiment 10** was designed to compare two concentrations of the homogenized phosphatidylcholine, and the commercial extender. High pressure homogenization was very efficacious in the dispersion of phosphatidylcholine vesicles and it was hypothesized that this may affect the concentration of phosphatidylcholine required to protect sperm during freeze/thawing. It has also been shown that the use of high molecular weight substances increase the survival of cells during ice crystal-free freezing (vitrification). Therefore, it was decided to examine the benefit of the addition of the colloidal substance, agar. However, the addition of agar did not improve sperm viability after freeze-thawing.

**Experiment 11** was designed to determine the benefit of the inclusion of the high molecular weight polymer, sodium hyaluronate, in protecting sperm frozen in homogenized extenders. Polymers such as sodium hyaluronate are believed to prevent ice crystallization and re-crystallization during freezing/thawing, resulting in vitrification (ice crystal-free freezing), which in turn, may minimize cell damage due to the formation of ice crystals. The same extender groups were compared to a homogenized extender group in which sodium hyaluronate had been added. The Tris-based extender with 5.0% homogenized phosphatidylcholine and 7.0% glycerol plus sodium hyaluronate and Pluronic F-68 was equally efficacious to AndroMed in the freezing of bovine semen.

**Experiment 12** was designed to investigate the use of the phosphatidylcholine extender in the freeze/thawing of European bison semen used for in vitro fertilization of bovine oocytes. An opportunity arose to utilize the phosphatidylcholine semen extender in the freezing of European bison semen which was to be used in an in vitro fertilization experiment in Spain. European bison semen was frozen in Triladyl or the phosphatidylcholine extender as described above. Semen from a Holstein bull was frozen as a Control. Following thawing, 1 million sperm were used to inseminate in vitro matured bovine oocytes in seven replicates. Zygotes were cultured for 24 h after insemination to determine fertilization rate. There was no significant difference in sperm motility between groups after dilution or freeze/thawing. Results of the in vitro sperm penetration assay with the Holstein bull semen frozen in Triladyl (Control; 62%) or with European bison semen frozen in Triladyl (43%) or Triladyl plus phosphatidylcholine extender (63%) were also not different. Results indicate that phosphatidylcholine of soybean origin can effectively replace egg yolk in the Triladyl extender for the cryopreservation of European bison semen suggesting many alternatives for the utilization of the potential final product. Indeed, we have successfully used this extender with other exotic species in the past.

## PART B – BOVITEQ AND UNIVERSITY OF GUELPH (JULY 2004 TO FEBRUARY 2007)

Project Objectives

The primary objective was to prepare a synthetic extender (without animal proteins) for

1. The cryopreservation of bovine semen,
2. The preservation (short and long-term) of porcine semen in liquid form,
3. The cryopreservation of porcine semen.

The specific objectives (milestones) of this overall project were:

1. To characterize how a solution containing plant lipids mediates bovine and porcine sperm function(s).

2. To determine the effect of plant lipids on bovine and swine sperm frozen in plant lipid extracts, and also for pig sperm processed for fresh insemination in a liquid extender.

3. Identify factors that affect the stability of a plant-lipid-based semen extender (shelf life of the extender) for bovine and swine sperm.

4. Evaluate efficacy of the extender as a cryopreservative for semen used for in-vitro fertilization and artificial insemination in the field for bovine and swine sperm.

It was decided that as the development of a bovine extender would likely be a more straight-forward task, the research thrust in the beginning should be towards that end. It was also felt that the swine extender would be relatively easier to develop with the knowledge and experience gained in the development of the bovine extender. Consequently, objectives and milestones were initially refocused toward the bovine extender. It was intended that the last 18 months of the contract would be devoted to the development and testing of the swine semen extender.

As with the initial studies at the University of Saskatchewan, the reference extender chosen was egg yolk-based as it is the most widely used lipoprotein supplement included in most semen extenders. Egg yolk is essentially an emulsion composed of approximately 50% water and 30% lipids. The lipid component is constituted of approximately 25% phospholipids and 75% triglycerides. Of all stable phospholipid +/- triglyceride suspensions, the literature reports different forms such as liposomes, micelles composed of only phospholipids or composed of triglycerides surrounded by phospholipids. Potentially, all of these forms are found in egg yolk. Therefore, we aimed to reproduce the lipid composition of egg yolk using non-animal sources of lipids. In addition, the University of Saskatchewan studies indicated that high-pressure homogenization of phosphatidylcholine preparations was the preferred approach to the production of liposomes for use in these studies; this was continued.

Preliminary work conducted at the University of Saskatchewan demonstrated that it was possible to replace egg yolk with phosphotidylcholine of plant origin during cryopreservation, and in early studies, we showed that lipids of soy lecithin origin maintained sperm survival during cold shock treatment (Figure 1). However, there were disadvantages of the product obtained from Sigma; it was composed of approximately 40% lipids of unknown nature. Even if we determined the nature of these lipids, variability of phosphatidylcholine concentration from batch to batch remained a problem as the manufacturer could not guarantee the purity of the product sold. In addition, Sigma did not assure that the product was 100% animal protein-free. Finally, the

percentage of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine (20:20:15) was very different than the ratio found in egg yolk. Therefore, Lipoid LLC was chosen as the manufacturer of the lipid source to be tested. The only disadvantage was the cost which was higher than for the Sigma product. The advantages were that our initial market analysis indicated that the higher price for the product would not have a significant impact on the actual cost associated with the extender preparation. In addition, Lipoid was a well established company which had been manufacturing and distributing their products for over 30 years. Lipoid's main market was various pharmaceutical products so they ensured that their products were well characterized and certified for use in humans; their products did not contain any animal proteins, no pathogenic agents, and the products' compositions were well characterized. Finally, Lipoid S75 ($Soy_{PC}$) was a lipid product of soybean origin with lipid compositions very close to what is found in egg yolk.

## BOVINE SYNTHETIC SEMEN EXTENDER

Figure 1 depicts the preliminary results showing sperm motility after subjecting extended bovine sperm to a 4°C cold shock treatment for 20 min. A cold shock treatment is a quick test done to sperm to determine the efficacy of the different extenders to help sperm survive temperature stress. The figure depicts survival rates after 1 and 4 h of incubation in the different extenders. Furthermore, these results demonstrate that of the motile sperm that were extended in various soy-based suspensions, all had comparable velocities (Figure 2) and sperm head displacements (Figure 3) as sperm extended in egg yolk.

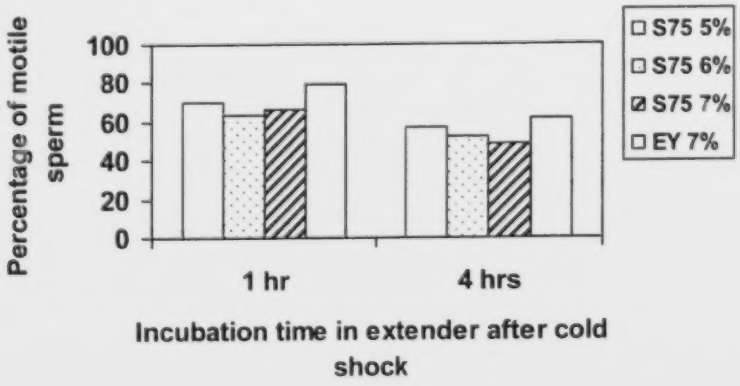



Figure 1. Sperm motility after a cold shock treatment for sperm cells extended in different concentrations of $Soy_{PC}$ lipid suspensions (S75) or egg yolk (EY)

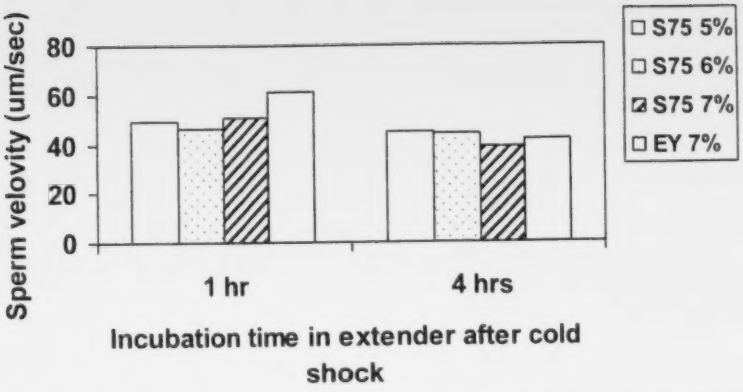



Figure 2. Sperm velocity following cold shock in different concentrations of $Soy_{PC}$ lipid suspensions (S75) or egg yolk (EY)

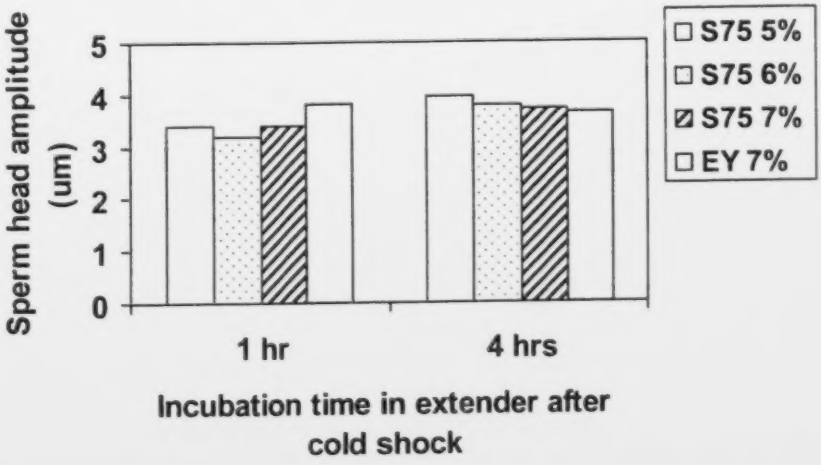



Figure 3. Motile sperm amplitude of lateral head displacement in different concentrations of $Soy_{PC}$ lipid suspensions (S75) or egg yolk (EY)

These preliminary results were very encouraging as sperm extended in $Soy_{PC}$ lipids at different concentrations had sperm motility parameters very close to that of sperm extended in egg yolk.

**Other extender preparations tested**

The aim of the following experiments was to target the optimum composition to be included in the final extender preparation. Over 50 different semen extender preparations consisting of different types of phospholipids, different types of triglycerides, buffers, preparation methods, etc., were evaluated in semen from four different bulls. Figures 4 to 6 describe the motility characteristics of sperm cryopreserved in the 13 different extenders (see Table 1) and then thawed and evaluated.

Briefly, sperm were washed twice in Tyrode's medium and incubated at 37.5°C in a controlled atmosphere (5% $CO_2$ in 100% humidified air). Motility was evaluated 1 and 6 h after thawing and incubation by CASA (Computer Assisted Sperm Analysis). Treatments 1 to 6 include phospholipids and canola oil at different concentrations. Treatments 7 to 12 include

phospholipids and coconut oil at different concentrations. In treatment 13, sperm were preserved in the egg yolk extender routinely used at the Centre d'Insémination Artificielle du Québec (CIAQ). From these results, it is clear that some of the preparations had beneficial effects on sperm following cryopreservation.

Table 1. Description of different semen extenders containing $Soy_{PC}$ (S75) and canola or coconut oil.

| Treatment | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lipid used | S75 + Canola Oil | | | | | | | S75 + Coconut Oil | | | | | CTRL |
| Nb of Homogenization | 5 | | | 20 | | | | 5 | | | 20 | | X |
| Final Lipid Percentage | 4 | 7 | 10 | 4 | 7 | 10 | 4 | 7 | 10 | 4 | 7 | 10 | 7 |

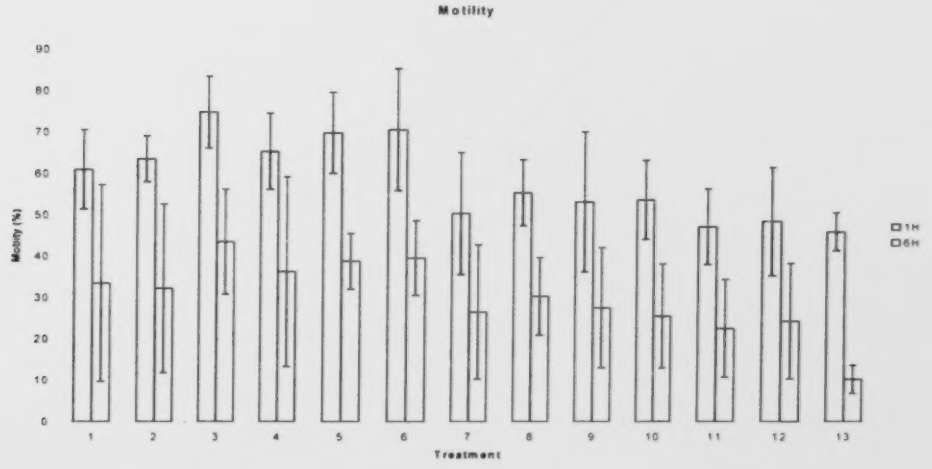



Figure 4. Percentage of motile bovine sperm 1 and 6 h after thawing of semen cryopreserved in 13 different extenders containing $Soy_{PC}$ and either canola oil or coconut oil or containing egg yolk (See Table 1 above).

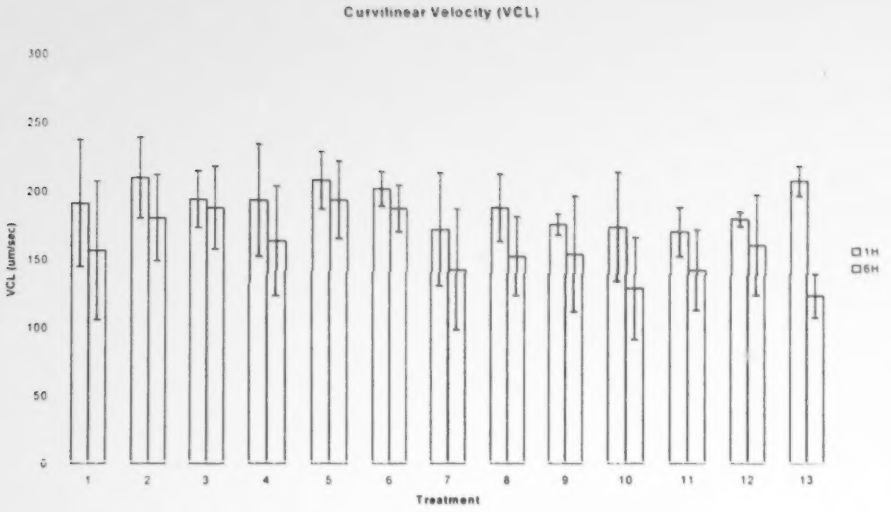



Figure 5. Curvilinear velocity of motile bovine sperm 1 and 6 h after thawing of semen cryopreserved in 13 different extenders containing $Soy_{PC}$ and either canola oil or coconut oil or containing egg yolk (See Table 1 above).

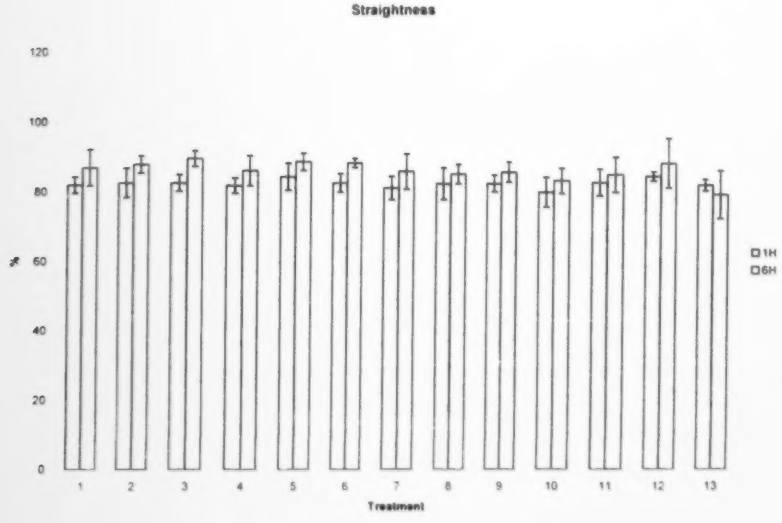



Figure 6. Percentage directional motility of bovine sperm 1 and 6 h after thawing of semen cryopreserved in 13 different extenders containing $Soy_{PC}$ and either canola oil or coconut oil or containing egg yolk (See Table 1 above).

**Development of a sub-optimal IVF system to be used to evaluate sperm fertility**

The sub-optimal IVF (in vitro fertilization) system was used as a tool to determine if bovine spermatozoa retain their ability to bind to and penetrate oocytes and fertilize them normally (i.e. very low rates of polyspermy) following cryopreservation. Firstly, we determined the effect of a shorter fertilization time ± heparin ± PHE (penicillamine, hipotaurine, epinephrine). Results are shown in Table 2. Both the reduction of the time of sperm and oocyte co-incubation and removal of heparin reduced the fertilization rates.

Table 2. Fertilization rates following IVF at various time intervals in heparin and/or PHE.

| Fertilization time | | | Fertilization rate | |
|---|---|---|---|---|
| | Heparin | PHE | Number | % |
| 22 h | 2 µg/mL | + | 36/41 | 87.8 |
| 6 h | 2 µg/mL | + | 21/54 | 38.9 |
| 6 h | - | + | 5/55 | 9 |
| 6 h | - | - | 2/59 | 3.4 |

In a second series of experiments, the impact of removing heparin for the longer (normal) incubation time, as well as the sperm concentration during fertilization, was evaluated. The results are shown in Table 3. A Percoll preparation method was compared to a washing procedure to select a motile sperm population used for IVF. We opted for a washing preparation method to have a representation of the whole sperm population after thawing; the washing method resulted in half the fertilization rate as the Percoll system. However, no heparin was added with this second method. Proper controls are included below. A reduction of the sperm number also reduced the fertilization rates as expected.

Table 3. Fertilization rates following IVF at various sperm concentrations.

| Sperm preparation | Fertilization time | Heparin | Sperm/oocyte Ratio | Fertilization rate | | Polyspermy rate | |
|---|---|---|---|---|---|---|---|
| | | | | No. | % | No. | % |
| Percoll | 20 h | 2 µg/mL | 10000:1 | 31/37 | 83.8 | 1/37 | 2.7 |
| Washing | 20 h | - | 10000:1 | 15/37 | 40.5 | 1/37 | 2.7 |
| Washing | 4 h | 2 µg/mL | 10000:1 | 6/34 | 17.6 | 0 | 0 |
| Washing | 4 h | 2 µg/mL | 5000:1 | 3/43 | 7.0 | 0 | 0 |

In a third series of experiments, the impact of reduced sperm concentration ± heparin was evaluated for a normal 20 h fertilization time. Results are shown in Table 4. As the polyspermy rate was high in the control treatments, we are uncertain as to how to interpret these results.

Table 4. Fertilizations rates following IVF at various sperm concentrations (20 h IVF).

| Sperm preparation | Fertilization time | Heparin | Sperm/oocyte Ratio | Fertilization rate | | Polyspermy rate | |
|---|---|---|---|---|---|---|---|
| | | | | No. | % | No. | % |
| Percoll | 20 h | 2 µg/mL | 10000:1 | 20/34 | 58.8 | 3/34 | 8.8 |
| Washing | 20 h | 2 µg/mL | 10000:1 | 25/41 | 61.0 | 6/41 | 14.6 |
| Washing | 20 h | 2 µg/mL | 5000:1 | 27/41 | 65.9 | 1/41 | 2.4 |
| Washing | 20 h | - | 10000:1 | 11/40 | 27.5 | 1/40 | 2.5 |
| Washing | 20 h | - | 5000:1 | 10/41 | 24.4 | 0 | 0 |

We have established using in vitro fertilization protocols that bovine semen cryopreserved in the soybean lipid (S75) + oil emulsifications (synthetic extenders) maintained its fertilizing ability post-thaw. Furthermore, these extenders maintained their integrity for at least 3 months. One-month-old synthetic extender performed as well as freshly prepared emulsifications in the cryopreservation of semen indicating the great versatility of the extenders. Preliminary data indicate that Bioxcell (IMV) and Andromed (Minitube) are made from mostly lipid preparations without any oil component.

**In vitro fertilization and embryo production**

One key milestone of this project was to ascertain that the semen cryopreserved in the synthetic extenders maintains its fertilizing ability post-thaw. In order to assess this, an IVF study involving three different bulls was undertaken. Semen was cryopreserved in the two different formulations as well as in the egg yolk extender currently used CIAQ. Ovaries were obtained from a local slaughterhouse. Collection and in vitro maturation (IVM) of cumulus-oocyte complexes were done as previously described (Bousquet et al. Theriogenology 1999;51:59-70). Very briefly, oocytes were washed in HEPES buffered Tyrodes's solution and matured in microdroplets of TCM-199 plus 10% foetal bovine serum with 5 ug/mL of pLH, 0.5 ug/mL pFSH and 1 ug/mL of estradiol-17β for 24 h at 38.5 °C, in a humidified atmosphere containing 5% $CO_2$. Fertilization was done using frozen-thawed, Percoll-separated semen, for 18 to 22 h in TL-Stock with 6 mg/mL of fatty acid-free BSA. On the following day (Day 1 of culture), presumptive zygotes were denuded and co-cultured with Buffalo rat liver cells in Menezo B2 medium with 0.4% fatty acid-free BSA for 36 h, and then with 10% estrous cow serum until Day 8 of culture in humidified atmosphere containing 5% $CO_2$ at 38.5°C. The percentage of zygotes, morula, blastocysts and hatched blastocysts produced were calculated based on the total number of oocytes inseminated for all groups (egg-yolk extender = 209 oocytes; 1:1.5 lipid formulation = 208 oocytes; 1:3 lipid formulation = 187 oocytes). Results are presented in Figure 7. It is clear from these results that both synthetic extender formulations can maintain the fertilizing potential of the semen as well as the egg-yolk extender after freeze-thaw; rates of embryonic development were similar in all groups.

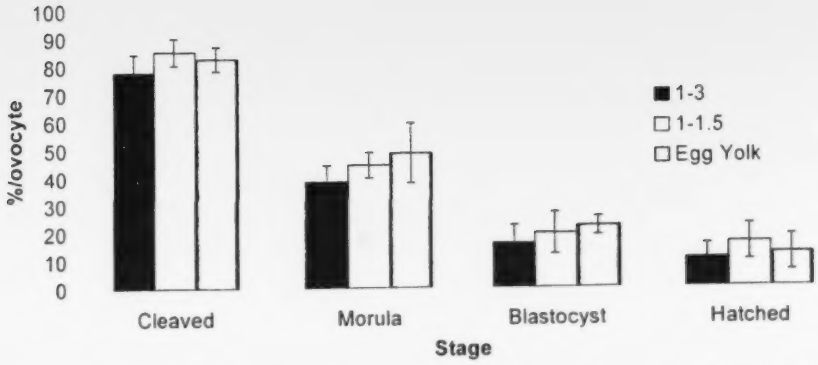



Figure 7. Semen from three different bulls was split and frozen in three different extenders. Extender 1:3 is a synthetic extender containing 2.5% phospholipids ($Soy_{PC}$) and 7.5% triglycerides (canola oil). Extender 1:1.5 is a synthetic extender containing 4% phospholipids ($Soy_{PC}$) and 6% triglycerides (canola oil). Semen was thawed and used to inseminate in vitro matured oocytes. The percentage of cleaved zygotes, morulae, blastocysts and hatched blastocysts was calculated using the total number of oocytes and determined after 2, 5, 8 and 9 days in culture, respectively. There were no differences among groups on any the days examined.

**Characterization of commercial canola oil**

As indicated earlier, most components of the extender developed in our labs are well characterized and of high purity. However, the canola oil component that is incorporated in the extender had not been fully characterized. This component is one element that distinguishes our product from the commercially available products and that gives the synthetic extender tested here major advantages. In order to make sure that the previous results can be reproduced, three different samples of canola oil were stocked for one month at 27°C in the dark. Following this stocking period, these samples, along with three fresh samples of canola oil, were sent (six samples were analyzed) for complete profiling of the fatty acid content. This way, we would be able to evaluate the batch-to-batch variation, as well as the effect of stocking this oil, with regards to the fatty acid composition. In the literature (Dmytryshyn et al., 2004 Bioresouce Technology 92:55-64), it is reported that canola oil contains the following fatty acids: 4% palmitic acid (16:0), 2% stearic acid (18:0), 62% oleic acid (18:1), 22% linoleic acid (18:2 or ω6) and 10% alpha linolenic acid (18:3 or ω3). Analysis of the fatty acid content of the six canola oil samples used in our studies is depicted in Figure 8. It is clear from these results that the oils used here are very similar in fatty acid content as reported in the literature and that the proportion of each fatty acid does not change after 1 month of storage. The small standard errors also indicate that there is little batch-to-batch variation. We are confident that all components used in the synthetic extender are of pure quality and can be maintained for some time.

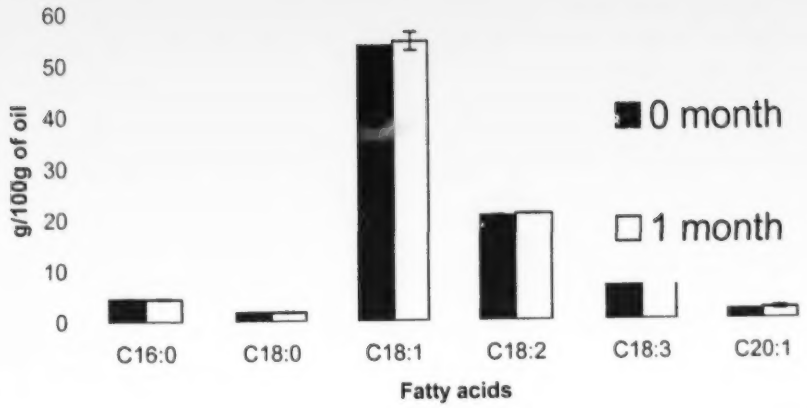



Figure 8. Three different batches of canola oil were stored for 1 month in the dark at 27°C. Additionally, three different batches were obtained (no storage time) and all six batches were then sampled for analysis. Total amount of conjugated fatty acids in the different samples was analyzed. Fatty acids present in "fresh" and one-month old canola oil did not differ.

**Particle sizing of the emulsifications after autoclaving and a 3-month storage**

In order to determine the shelf life of the lipid emulsifications and to verify that autoclaving does not cause adverse effects, three batches of the two most efficacious lipid emulsifications (1:1.5 and 1:3 lipid to oil ratio) were produced. A sample of each emulsification was autoclaved and all emulsifications were stored in the refrigerator (4°C). Particle size of these emulsifications was monitored over 3 months using photon correlation spectroscopy (PCS; Malvern instrument) in the Department of Pharmacology of University of Montreal. The results of these analyses are presented in Figure 9. It is clear from these results that both emulsifications tested (1:1.5 and 1:3) maintained their particle size over the three-month period. Furthermore, autoclaving these samples did not modify the size of these particles.

**Efficiency of the emulsifications over a one month storage**

The stability of the emulsifications was tested by cryopreservation of freshly ejaculated bovine sperm using three different bulls. Two cryopreservation sessions were performed: the first using the freshly fabricated emulsifications and the second using the same emulsifications that were stored at 4°C for one month. In both sessions, the same three bulls were used. Briefly, sperm were thawed and washed twice in a Tyrode medium and incubated at 37.5°C in a controlled atmosphere. Motility was evaluated using a CASA 1 and 6 h after thawing. Results are presented in Figure 10. These results demonstrate that the two synthetic emulsifications tested (whether autoclaved or not) conserve the overall sperm motility when compared to the standard egg-yolk extender. We can see that all synthetic extenders resulted in a minimum of 10% more motile sperm when compared to the egg-yolk extender 1 h after thawing (Figure 10A). After 6 h of in vitro incubation, the synthetic extenders still out-performed the egg-yolk extender as the overall motility remained high (Figure 10B). Similar findings were observed when the percentage of progressive motile sperm was analyzed; the synthetic extenders resulted in higher percentages of progressively motile sperm 1 h (Figure 10C) and 6 h (Figure 10D) after thawing.

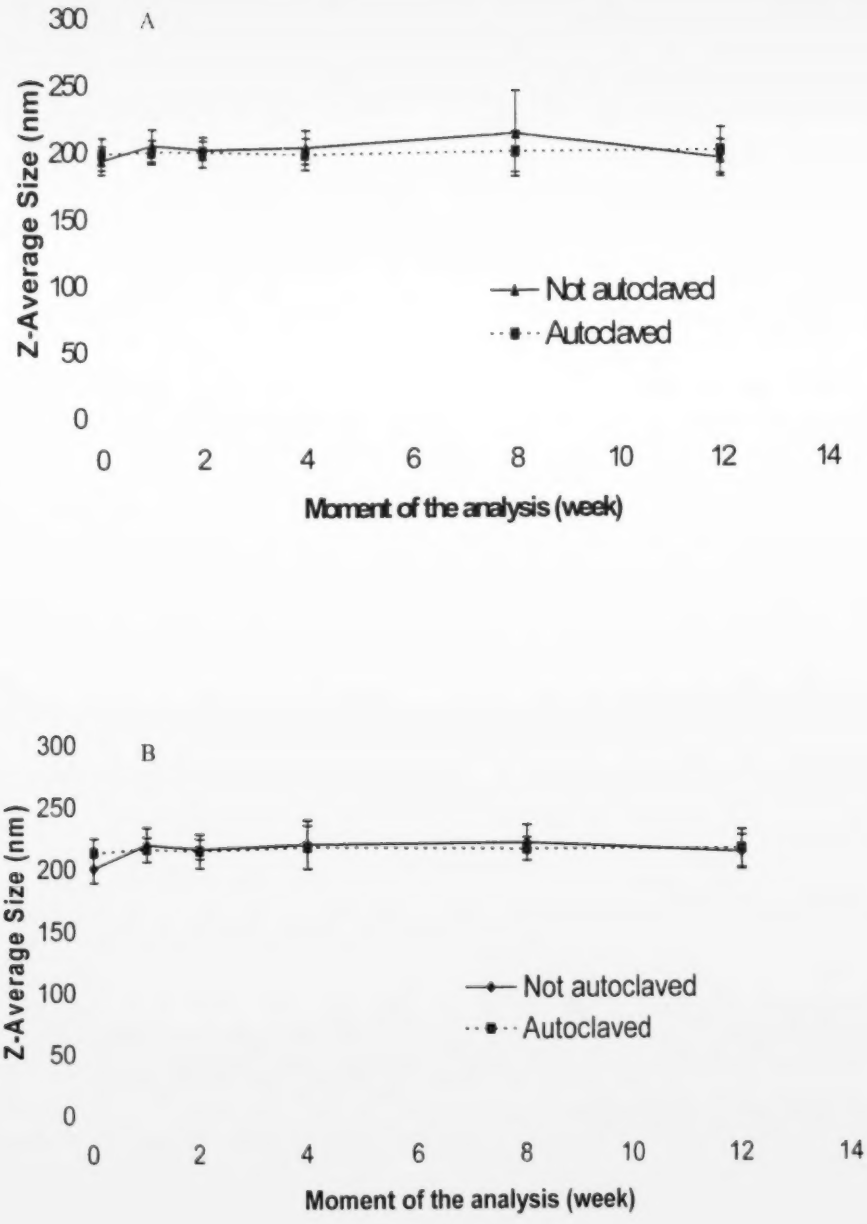



Figure 9. Z-average size of the particles from the lipid emulsifications 1:1.5 (A) and 1:3 (B). Lipid emulsifications were prepared on week 0 and some were autoclaved. They were then stored at 4°C in the dark for the period of evaluation. Particle size assessment was done by photon correlation spectroscopy after 0, 1, 2, 4, 8 and 12 weeks.

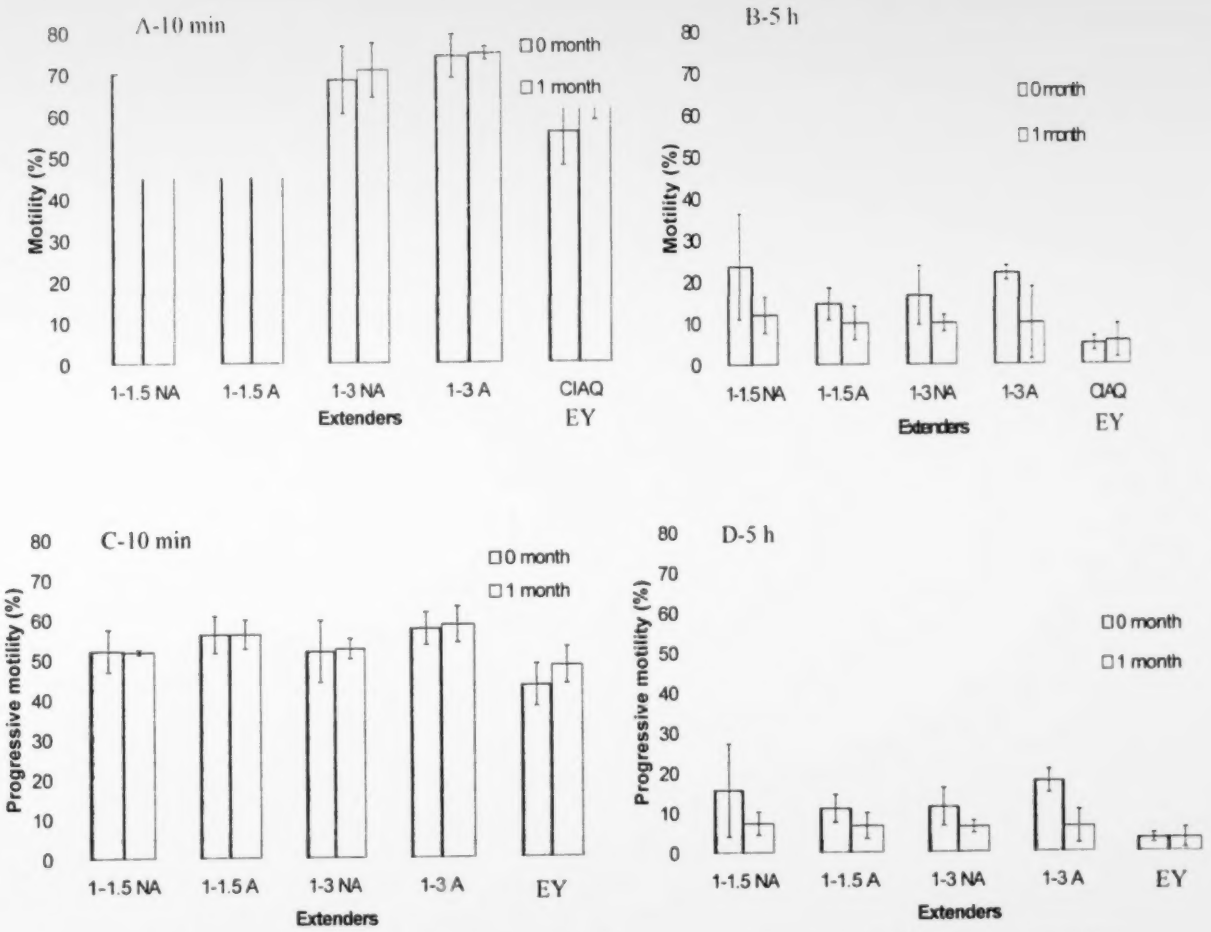



Figure 10. Comparison of motility post-thaw for semen cryopreserved in different extenders. Five different extender preparations were tested: non-autoclaved synthetic 1:1.5 lipid emulsification (1-1.5 NA), autoclaved synthetic 1:1.5 lipid emulsification (1-1.5 A), non-autoclaved synthetic 1:3 lipid emulsification (1-3 NA), autoclaved synthetic 1:3 lipid emulsification (1-3 A), and egg yolk extender prepared at CIAQ (EY). Synthetic extenders were produced using fresh canola oil or oil that had been stored for one month. Note that for the EY, fresh extender was prepared in all treatments presented here. Sperm motility is analyzed by CASA after 10 minutes (panels A and C) and 5 h (panels B and D) in vitro incubation.

**What is Bioxcell? What is Andromed?**

Another aspect of the present project consisted of comparing our formulations with already available synthetic extenders, specifically Bioxcell from IMV and Andromed from Minitüb. To understand better the composition of these extenders, the particle size of both extenders was evaluated by PCS. It appears that both extenders have smaller particle sizes (Bioxcell = 120 nm and Andromed = 138 nm) when compared to our extender (approximately 200 nm). Based on the initial work we have done comparing various combinations of phospholipids and oils, we believe that extenders from Bioxcell and Andromed are probably composed of mostly, if not entirely, phosphatidylcholine liposomes.

**In summary**, studies over the first year identified two out of several formulations that were suitable for future studies. They were selected based on their performance at maintaining viability, motility and acrosome integrity. Semen preserved in both of these preparations

produced a rate of embryo production that did not differ from semen preserved in the egg yolk extender; however, the 1-1.5 lipid preparation was considered to have a slight edge over the other preparations. It was also shown that the synthetic formulations have a shelf life of more than 3 months. During the final year, efforts concentrated on comparing semen preserved in the 1-1.5 synthetic extender against semen preserved in egg yolk in terms of in vitro intracellular characteristics and in vivo fertility. In addition, an extended experiment over an 8-month period was conducted to evaluate the shelf life of the 1-1.5 lipid preparation.

**In vivo fertilization assay in superstimulated cows**

In this setting, artificial insemination was performed on superovulated cows. Embryos and unfertilized ova were recovered by nonsurgical embryo collection procedures to determine the percentage of fertilization. This test is considered in house as a semi-quantitative evaluation but was performed to determine if in vivo fertilization could be obtained with semen preserved in the synthetic extender. Although the semen preserved in the synthetic extender had already proven its fertilizing ability in vitro, verifying its in vivo fertilizing potential in house was a crucial preliminary to the conducting of a field trial.

Briefly, five different cows were superstimulated and matched with a single bull. Two ejaculates were collected from each bull and each ejaculate was split and diluted in either the synthetic or the egg yolk extender currently used at CIAQ. Each cow was superstimulated twice: inseminated once with the synthetic extender and once with the egg yolk extender. Each superovulation/insemination run comprised two inseminations by the CIAQ inseminators: one with the first ejaculate, one with the second ejaculate. Embryos and unfertilized ova were recovered nonsurgically 8 days post-insemination. The percentage of fertilized ova was obtained by dividing the number of embryos (fertilized) recovered by the total number of ova and embryos recovered. Table 5 presents the results obtained (paired analysis) for the synthetic and the egg yolk extender.

Table 5. Fertilization rates obtained in five superstimulated cows inseminated with semen from five bulls cryopreserved in the synthetic or egg yolk extender.

| Extender | Bulls | Number of oocytes and ova recovered nonsurgically | Fertilization rate (%) |
|---|---|---|---|
| Synthetic | 1 | 19 | 68.4 |
| Synthetic | 2 | 5 | 100 |
| Synthetic | 3 | 8 | 100 |
| Synthetic | 4 | 7 | 100 |
| Synthetic | 5 | 9 | 33.3 |
| Egg Yolk | 1 | 7 | 100 |
| Egg Yolk | 2 | 11 | 100 |
| Egg Yolk | 3 | 9 | 88.9 |
| Egg Yolk | 4 | 1 | 100 |
| Egg Yolk | 5 | 7 | 100 |

Although insufficient numbers of cows were inseminated in this experiment to generate enough data for elaborated statistical analysis, results demonstrated that in vivo fertilization rates with the synthetic extender were similar to the egg yolk extender. Most of the inseminations done with semen cryopreserved in the synthetic extender generated 100% fertilization rates; a 68.4% fertilization rate was obtained in a cow that produced a total of 19 ova and embryos with 12 embryos in a single collection. The synthetic extender was considered acceptable for a field trial. Furthermore, a field trial was considered necessary to quantify the fertility of the semen preserved in the synthetic extender.

**Assessment of the shelf life of non-autoclaved and autoclaved versions of the lipid preparation – particle sizing**

In order to assess the physical properties and the stability of the lipid preparations and in order to ensure that autoclaving does not harm the quality of the emulsification, photon correlation spectroscopy was used to perform particle sizing on the non-autoclaved and autoclaved versions of the phospholipid-canola oil preparation immediately after preparation, 3 months and 8 months later. The results shown on Figure 11 are consistent with previous data obtained for previous batches of lipid emulsification prepared. The average particle size was 189.4 nm, remaining unchanged through an 8 month period, whether the emulsification was autoclaved or not.

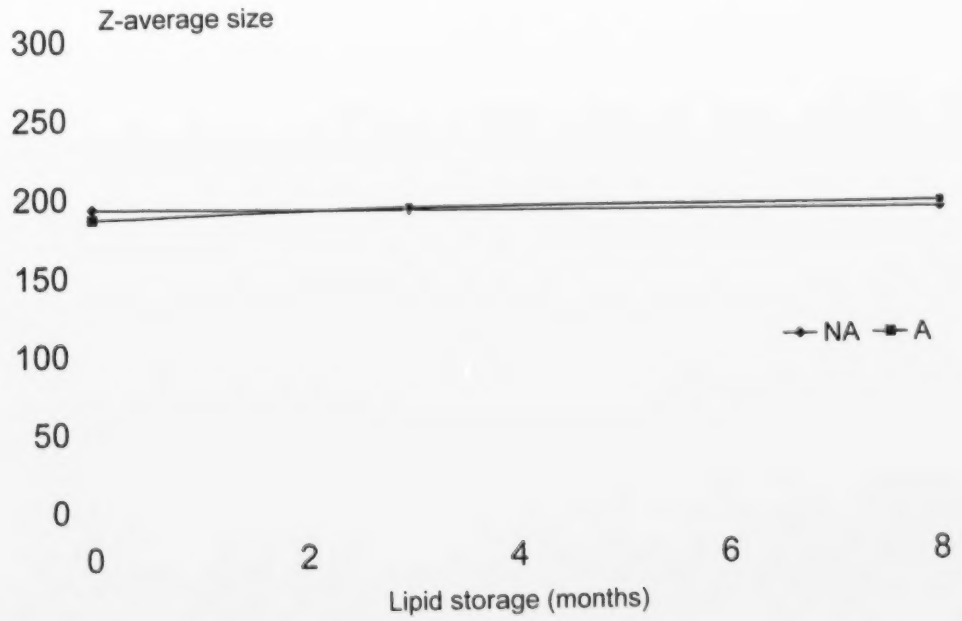



Figure 11. Particle size of the non-autoclaved (NA) and autoclaved (A) lipid emulsifications used to prepare the synthetic extender after 1 day (month 0), 3 months and 8 months of storage at 4°C in the dark. The average particle size was 189.4 nm, remaining unchanged through an 8 month period, whether the emulsification was autoclaved or not.

**Assessment of the shelf life of non-autoclaved and autoclaved versions of the lipid preparation – evaluation of the sperm functions**

In order to verify the stability of the oil-phospholipid emulsification in water, a batch of phospholipid-canola oil preparation was emulsified following the procedure described above and divided into two samples. One of the samples was autoclaved and the other was left non-autoclaved. Both samples were placed in the refrigerator (4°C). Ten (10) bulls were chosen because they could be collected over an 8-month period (proven bulls). One ejaculate from each bull was collected the day after the lipid emulsifications were stored in the refrigerator (month 0). Each ejaculate was split and diluted at equal concentrations in the standard synthetic extender made from the non-autoclaved lipid preparation or in the synthetic extender made from the autoclaved lipid preparation or in the egg yolk extender prepared by CIAQ. Semen was then cooled and frozen according to standard CIAQ industrial methods. Two identical freezing sessions were performed again at 3 months (month 3) and 8 months (month 8) with the same lipid preparations maintained in the refrigerator at 4°C for that time. At the end of this process, nine treatments (three extenders and three collection dates) were obtained for each bull.

One bull was slaughtered 5 months into the experiment so data were recovered from the nine remaining bulls. Semen from each bull, nine treatments and two straws per treatment, was thawed each day. Straws of the same treatment were pooled two by two and were suspended in 2.5 mL of Sperm-Talp which is a medium that mimics the bovine female oviductal fluid, and semen was handled in that medium for further steps. Semen was centrifuged at 250 x g for 5 min. and the pellet was resuspended and washed again in 2.5 mL of Sperm-Talp medium. A small sample of washed semen was used to assess motility and concentration. For each of the nine treatments, six million spermatozoa were then incubated in a controlled atmosphere (38.5°C and 5% $CO_2$) for 30 min in a total volume of 1 mL in the presence of Indo-1 AM and BCECF AM, two dyes used to measure relative intracytoplasmic calcium content and pH, respectively. Then, excess dye was removed by washing and centrifuging at 250 x g for 5 min. A sample of 1 million spermatozoa was taken from each of the nine tubes, suspended in a total volume of 500 µL containing PI and PE-PNA, incubated for 10 min at 35° and analysed by flow cytometry using a BD bioscience LSR II flow cytometer. PI and PE-PNA are two dyes used to assess cytoplasmic and acrosomal membrane integrity, respectively. The remaining 5 million spermatozoa were suspended in a total volume of 250 µL at a concentration of 20 million/mL and were incubated for an additional 4 h in a controlled atmosphere (38.5°C and 5% $CO_2$). At the end of the incubation, a 50 µL sample from each of the treatments containing 1 million spermatozoa was added to a 450 µL Sperm-Talp tube already containing PI and PE-PNA. The resulting 500 µL tubes were incubated at 35°C for 10 min and analysed by flow cytometry. A sample from each treatment is also used to assess motility.

The results show that the motility was very comparable in the three extenders from the start: the standard non-autoclaved version of the synthetic, the autoclaved version and the egg yolk extender (Figures 12-15). After 3 months of storage, the two versions of the synthetic extender still compared to the freshly prepared egg yolk extender. The only parameter that seemed affected by the 3 month storage was sperm velocity after 6 h of incubation. After 8 months of storage, the non-autoclaved version was still capable of preserving sperm motility through cryopreservation. Nonetheless, the 8 month-old autoclaved version yielded reduced motility parameters compared to the freshly-made egg yolk extender or the standard (non-autoclaved) synthetic extender stored for 8 months.

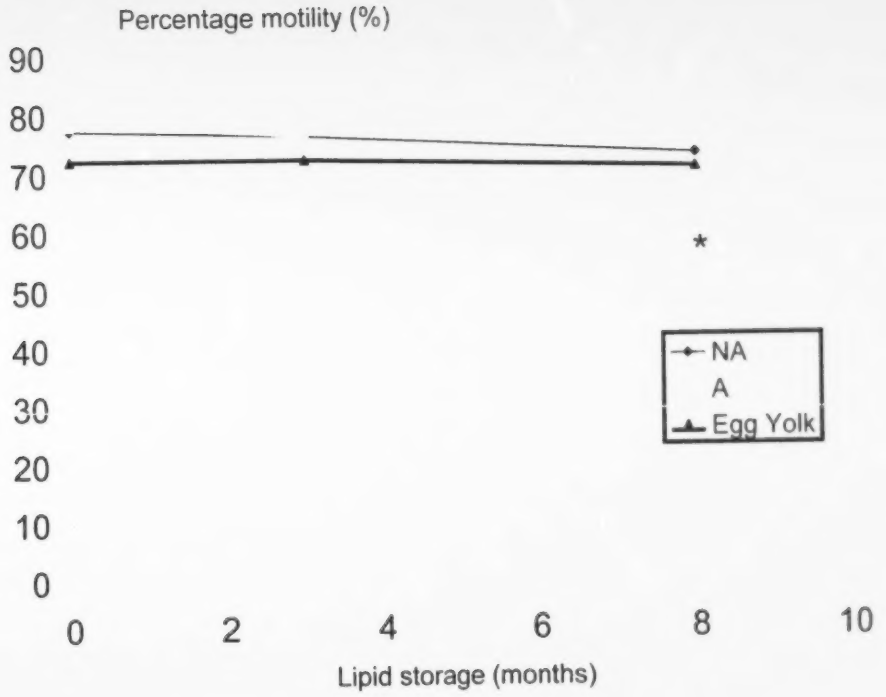



Figure 12. Percentage motility 1 h post thaw of sperm cryopreserved in the standard non-autoclaved or autoclaved versions of the synthetic or the egg yolk extender.

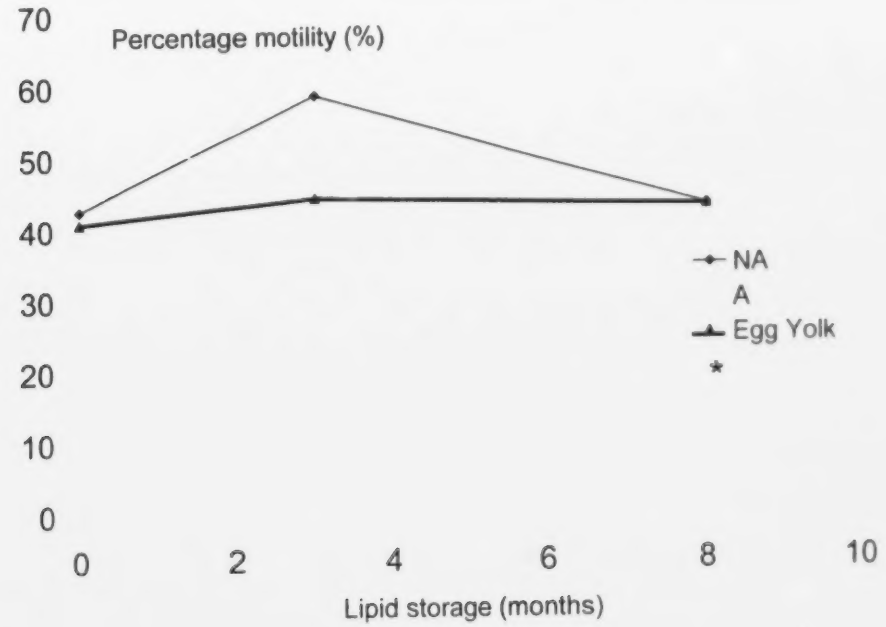



Figure 13. Percentage motility 6 h post thaw of sperm cryopreserved in the standard non-autoclaved or autoclaved versions of the synthetic or the egg yolk extender.

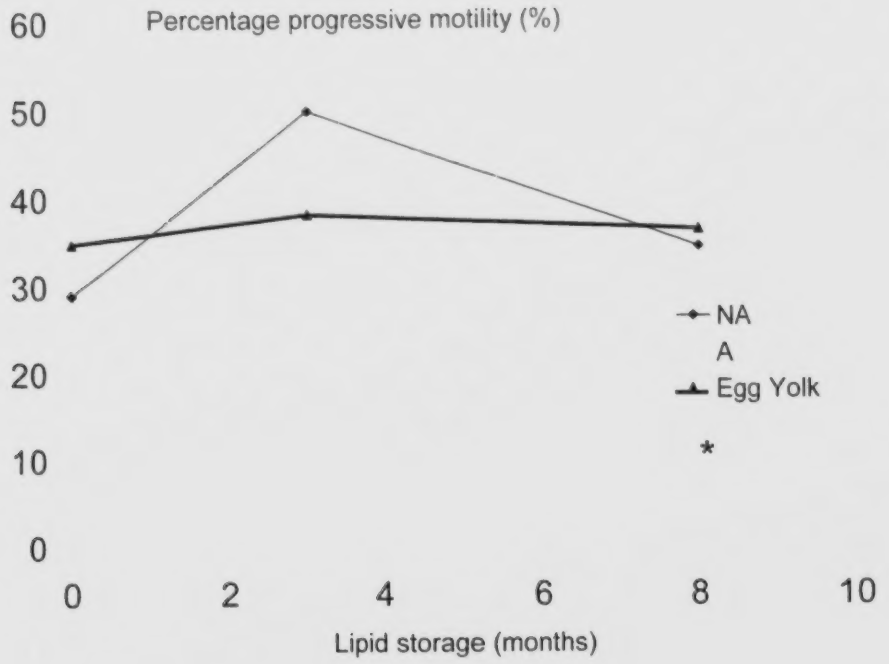



Figure 14. Percentage progressive motility 6 h post thaw of sperm cryopreserved in the standard non-autoclaved or autoclaved versions of the synthetic or the egg yolk extender.

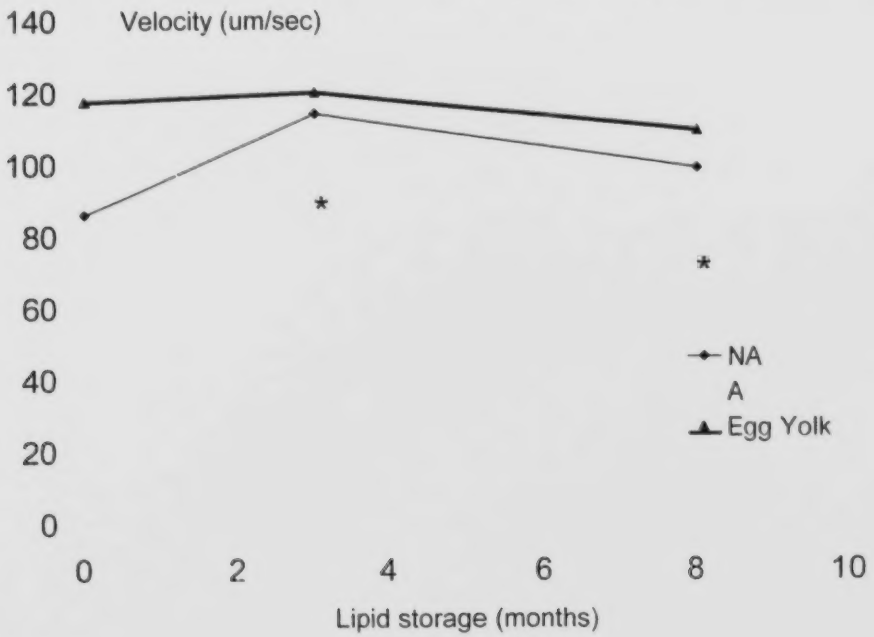



Figure 15. Average path velocity of sperm 6 h post thaw of sperm cryopreserved in the standard non-autoclaved or autoclaved versions of the synthetic or the egg yolk extender.

Although the freshly-made egg yolk extender compared to the synthetic extender in terms of motility parameters, it did not compare to the synthetic in terms of maintaining viability of the semen (Figures 16 and 17), integrity of the acrosome (Figure 18 and 19) or maintaining low intracytoplasmic pH (Figure 20). Regardless of the time of incubation, all bulls had a lower amount of dead spermatozoa after cryopreservation in the fresh or 3 month-old synthetic extender than in the freshly prepared egg yolk extender. After 8 months of storage, the non-autoclaved synthetic and the fresh egg yolk extenders were comparable. Again, regardless of the incubation time, all bulls had lower rates of acrosomal reaction when semen was preserved in the synthetic extender. Moreover, relative intracellular pH was lower in all bull's semen samples, except one, preserved in the synthetic extender, when assessed after 6 h of incubation. Intracellular pH increases as bovine sperm capacitate; results are in perfect agreement with the lower percentages of acrosomal reaction.

In summary, the autoclaved version was comparable to the non-autoclaved version until 3 months of storage. After 8 months of storage, data obtained with the autoclaved version indicated reduced sperm function compared to the non-autoclaved version.

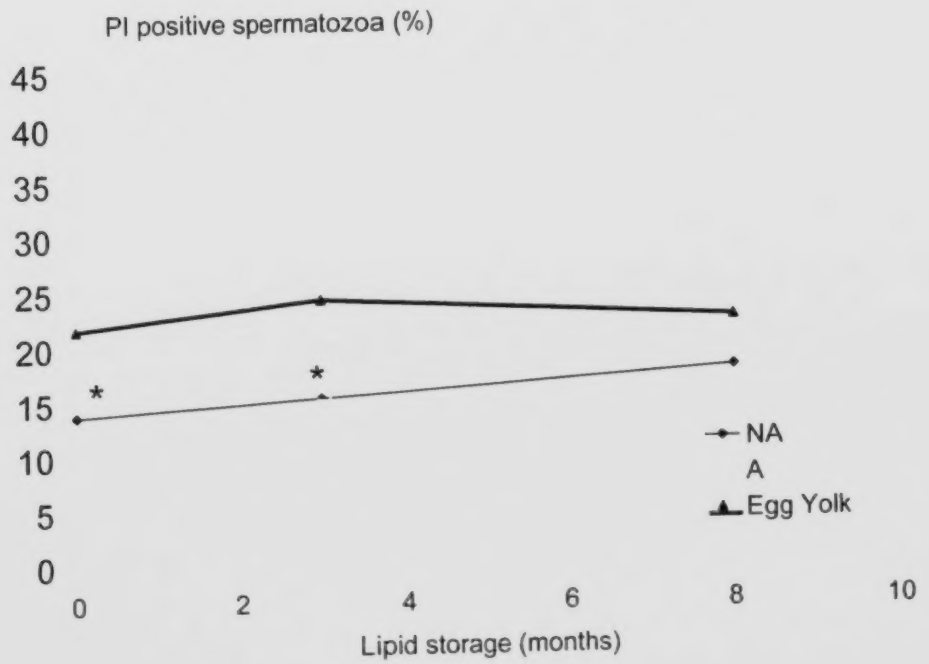



Figure 16. Percentage mortality 2 h post-thaw of sperm cryopreserved in the standard non-autoclaved or autoclaved versions of the synthetic extender or the egg yolk extender as assessed by propidium iodide (PI) permeability.

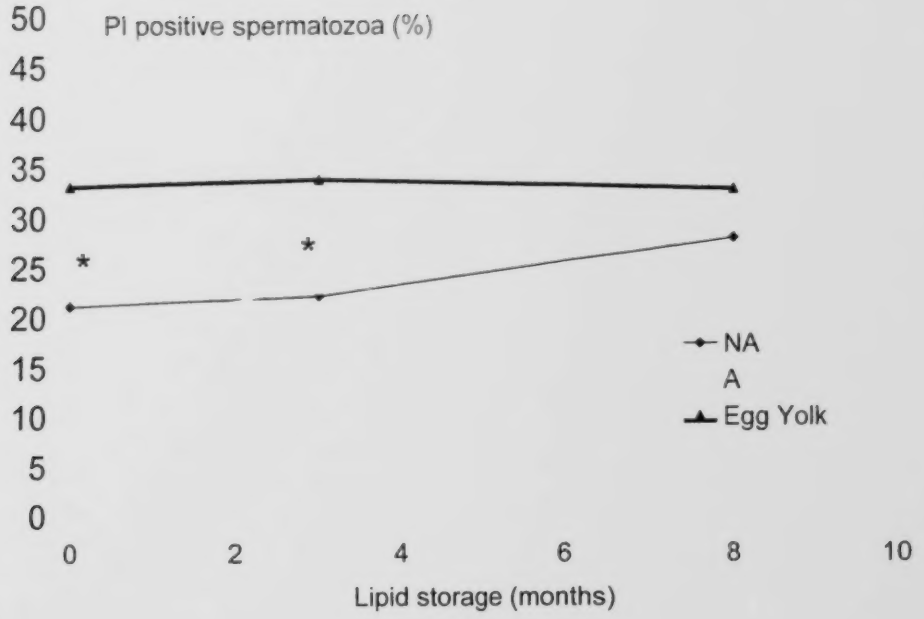



Figure 17. Percentage mortality 6 h post-thaw of sperm cryopreserved in the standard non-autoclaved or autoclaved versions of the synthetic extender or the egg yolk extender as assessed by propidium iodide (PI) permeability.

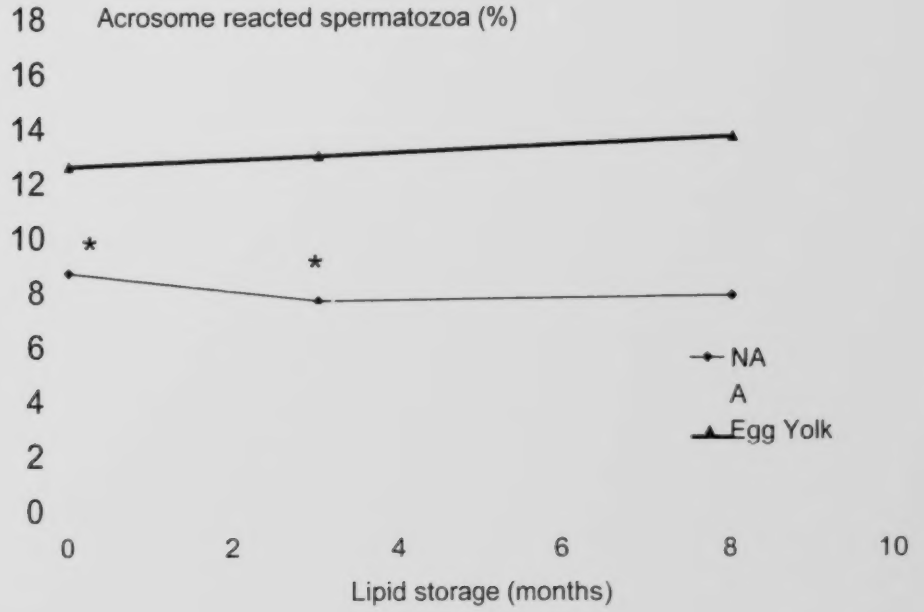



Figure 18. Percentage acrosome-reacted sperm 2 h post-thaw following cryopreservation in the standard non-autoclaved or autoclaved versions of the synthetic extender or the egg yolk extender as assessed by peanut agglutinin binding.

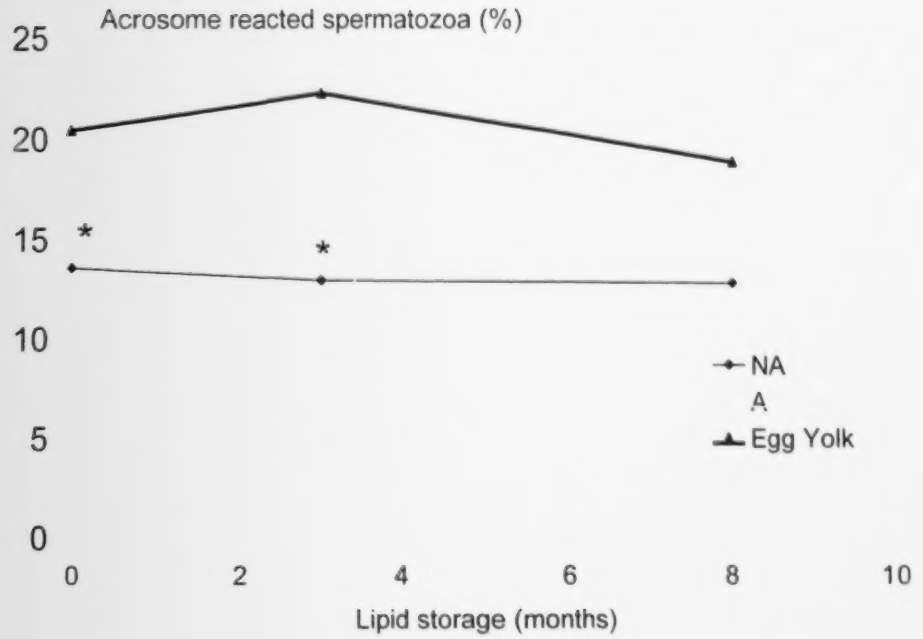



Figure 19. Percentage acrosome-reacted sperm 6 h post-thaw following cryopreservation in the standard non-autoclaved or autoclaved versions of the synthetic extender or the egg yolk extender as assessed by peanut agglutinin binding.

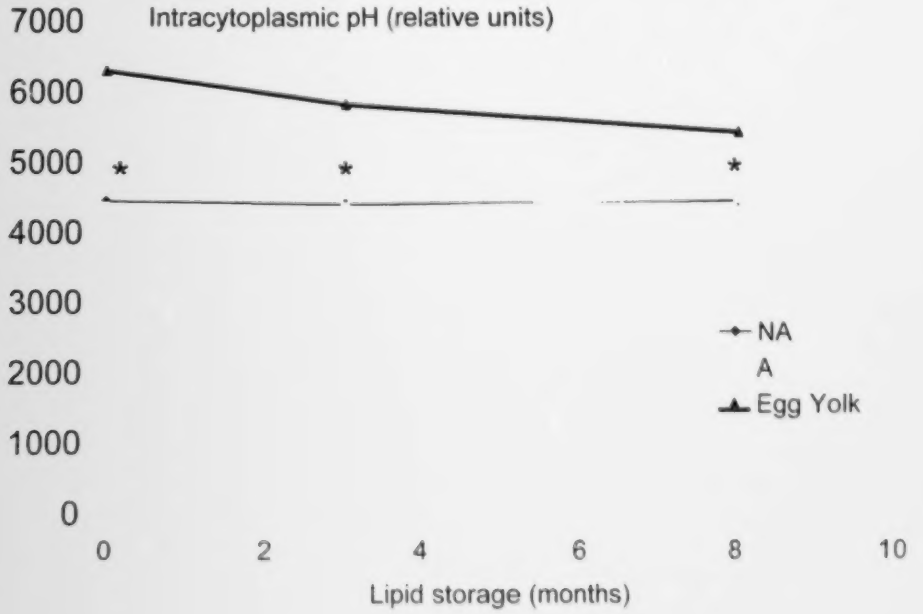



Figure 20. Relative intracytoplasmic pH 6 h. post-thaw of sperm cryopreserved in the standard non-autoclaved or autoclaved versions of the synthetic extender or the egg yolk extender as assessed by BCECF-AM fluorescence intensity.

Overall, control data obtained with the egg yolk extender constituted a real challenge for the synthetic extender to surpass. Semen samples displaying more than 40 - 60% viability were routinely obtained after cryopreservation, two washing steps, incubation with dyes, one additional washing step, and incubation, demonstrating the high quality of the semen handling. These data suggest that the synthetic extender was superior to the egg yolk extender in many aspects of sperm function, even after 8 months of storage. This was also true for the autoclaved version, although the advantage was reduced between 3 and 8 months of storage.

**Field trial comparing the synthetic extender and the egg yolk extender**

In an attempt to compare the egg yolk and the synthetic extender on a quantitative basis, four bulls in high demand for their genetic potential, were collected twice on two different days. Each ejaculate was split and diluted in the 1-1.5 synthetic extender (non-autoclaved) or the egg yolk extender as a control. Semen was always frozen by technicians at CIAQ using industrial procedures. A total of 600 AI doses were distributed as canes of three straws (two synthetic and one egg yolk) to over 70 different farms in the province of Québec. Inseminations were done by the CIAQ inseminators according to industrial procedures. Pregnancy rates were determined by ultrasonography 33 days (± 3 days) post-insemination.

The results obtained so far show that there was no difference in fertility between the semen preserved in the synthetic extender or the semen preserved in the egg yolk extender ($P = 0.25$). Additional data will be obtained during the next month and will be included in the analysis to maximise its reliability. So far the pregnancy rates obtained with the synthetic and the egg yolk extenders were 47% and 57%, respectively (results from 30% of the inseminations reported).

**Evaluation of hyaluronan supplementation of the synthetic extender**

It was hypothesized that hyaluronan (MAP-5) would help to preserve sperm function during cryopreservation. Sperm from three different bulls with different fertility levels were cryopreserved in the synthetic extender supplemented with 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL or PBS. Standard synthetic extender (without hyaluronan) was used as a control. Semen was cryopreserved following regular procedures at CIAQ and thawed for sperm function assessment. Semen was washed, marked and evaluated by the methods described above. All parameters assessed during this preliminary experiment indicate no differences in samples supplemented with hyaluronan as compared to the synthetic extender.

**Evaluation of Oxyfree as a synthetic extender supplement**

Oxidative stress is believed to be one of the major factors causing sperm damage during cryopreservation. Effectively, reactive oxygen species are likely to affect sperm membranes by phospholipid oxidation. This experiment was designed to determine if a commercial antioxidant cocktail would improve the efficacy of the synthetic extender. Semen from five different bulls with varying fertility was preserved in the synthetic extender supplemented with Oxyfree or without Oxyfree (control). Semen was processed as described above to evaluate in vitro parameters of sperm function.

Results indicate that the supplementation of the synthetic extender with Oxyfree improved its efficacy in the preservation sperm function. Semen from all bulls included in the analysis showed improved viability (Figure 21) and reduced intracytoplasmic calcium concentrations after 6 h of incubation in Sperm-Talp medium (Figure 22). In addition, semen from all bulls included in the analysis had improved progressive motility (Figure 23) and sperm velocity

parameters (Figure 24) after 1 and 6 h of incubation in Sperm-Talp medium. Semen preserved in the two different extenders was comparable for the other parameters assessed.

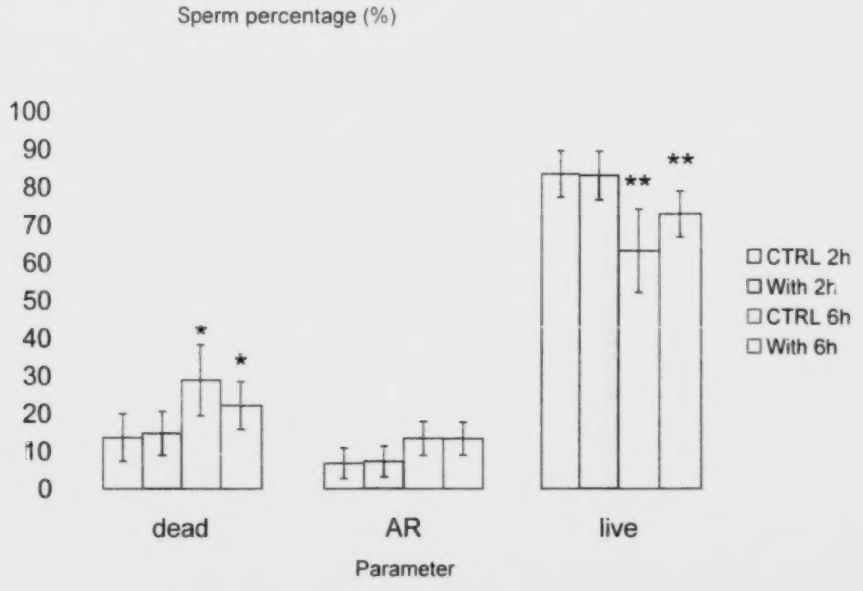



Figure 21. Percentages of dead, acrosome-reacted and live sperm, respectively assessed by PI exclusion, PNA binding and BCECF inclusion 2 and 6 h post thaw in the presence (with) or the absence (CTRL) of Oxyfree. * and ** - differences significant at 6 h ($P<0.05$)

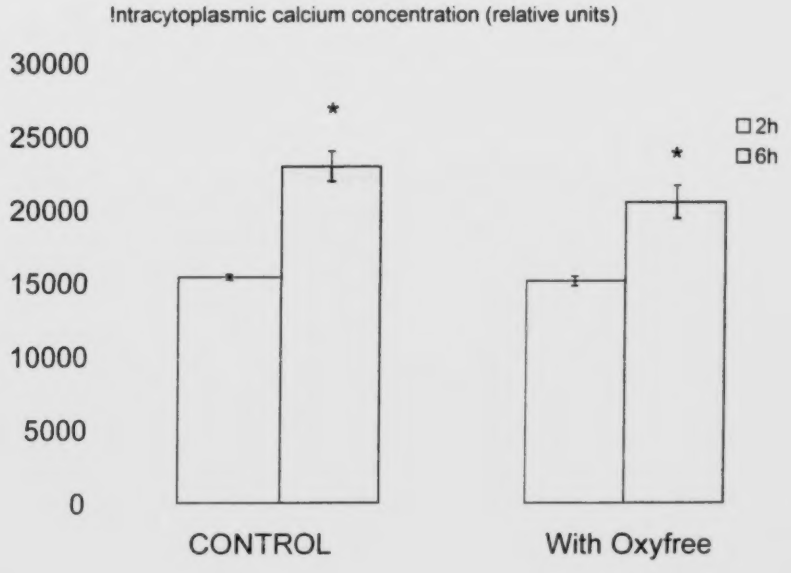



Figure 22. Relative intracytoplasmic calcium concentration 2 and 6 h post thaw in the presence of (with) or absence (CONTROL) of Oxyfree. *Significantly different at 6 h ($P<0.05$).

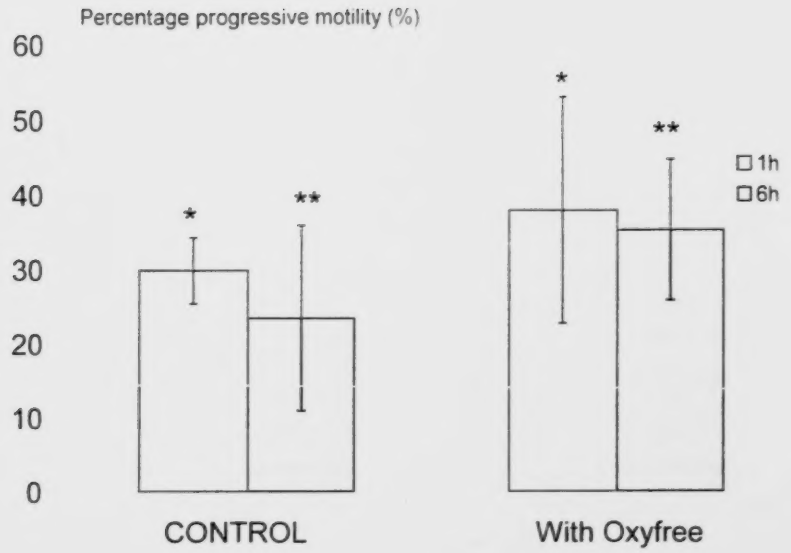



Figure 23. Sperm percentage progressive motility 1 and 6 h post-thaw in the presence (with) or absence (CONTROL) of Oxyfree. * and **Differences between preparations and times ($P<0.05$).

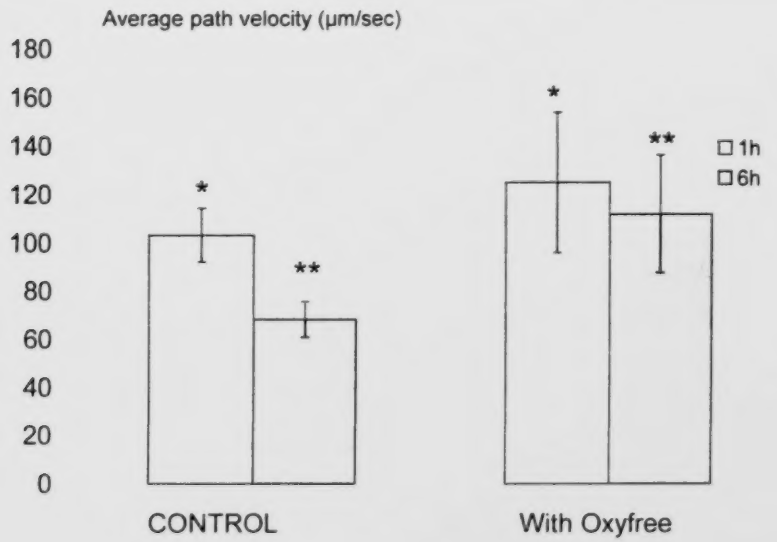



Figure 24. Sperm average path velocity 1 and 6 h post-thaw in the presence (with) or absence (CONTROL) of Oxyfree. * and **Differences between preparations and times ($P<0.05$).

This preliminary experiment indicates that Oxyfree could be a very efficacious supplement to the synthetic extender. Considering the improvement of in vitro sperm functions obtained on every bull, the hypothesis that the Oxyfree could improve the field fertility of the semen preserved in the synthetic extender is logical.

### Evaluation of fertility proteins as a synthetic extender supplement

As highlighted in the project proposal, it was also intended to test different fertility proteins that had developed with other collaborators. Overall, it has not been possible to demonstrate that fertility proteins in the different extender preparations effectively increased sperm survival, motility or fertility after freeze-thaw. Therefore, the addition of fertility proteins in the synthetic extender will not be pursued further until the work of collaborators demonstrate the activity of such proteins in current extender preparations.

## PORCINE SYNTHETIC EXTENDER

Dr Mary Buhr from the University of Guelph supervised the porcine semen research done in these studies. Identification and initial tests were done to select the lipids that are normally found on porcine sperm head membranes to be included in the extender. This was based on data included in the two patents issued to Dr. Buhr's group that were secured in this interinstitutional collaboration. As the work described in the patents was initially done using lipids from flax sources, preliminary work was done using this source of lipid. However, based on the progress with bovine semen, subsequent experiments utilized lipids originating from soybean lethicin. This constitutes a major advantage of the combined efforts of the three research teams. Furthermore, the University of Guelph possesses the expertise to evaluate the quality of the extenders produced using electron microscopy which was also important for the extenders produced for bovine use. Soy-based extenders developed for bovine semen were shared with the group from Guelph to test on porcine semen.

We established that phosphatidylcholine of soybean origin forms liposomes of a consistent size when produced by high-pressure filtration. This size was stable for at least 24 h, indicative of acceptable storage time. The liposomes fused with swine sperm, actually integrating into the sperm membranes and not simply adhering to the outside of the sperm. At low concentrations, all sperm took up the lipids rapidly and the fusion was stable over at least 24 h. A preliminary trial examining only one concentration of one simple lipid composition indicated that the lipids did not harm, and may have enhanced the percentage of motile sperm over 48 h of storage, appearing to actually increase the percentage of viable sperm.

### Liposome characteristics

Three types of liposomes were prepared: soybean phosphatidylcholine from Lipoids Inc ($Soy_{PC}$); Select Lipids (SL) from the Guelph recipe, and a 50:50 (mol:mol) mix of $Soy_{PC}$ and SL. Each was solubilised in chloroform:methanol (2:1, vol:vol), dried under a stream of nitrogen gas followed by 1 h in a vacuum dessicator, and then solubilised in the simple porcine (Beltsville) extender BTS (60°C, 60 sec, vortex 30 seconds; repeated three times). The lipid suspension was placed in the chamber of a syringe filter housing fitted with a 0.1 μm Nucleopore Polycarbonate Membrane (Whatman Inc. Clifton, New Jersey). The filter housing was attached to a nitrogen gas source charged to 500 psi that was opened to force the solubilised lipid through the filter. All liposomes were assessed within 30 min of preparation by Dynamic Light Scattering (DLS) with a Malvern Microfluidizer; model M-110-S (Malvern Instruments, Herrenberg, Germany), which provided six independent measures and then used an internal algorithm to convert the light scattering characteristics into liposome diameter (Dalgleish & Hallett, 1995) for each replicate batch of liposomes. Liposomes were stored under nitrogen gas at 5°C overnight and re-measured 24 h after preparation.

Liposomes were extremely consistent in shape and size, and did not change in size after 24 h of storage. The $Soy_{PC}$, SL, and SL-$Soy_{PC}$ being 60.3 ± 4.1, 61.1 ±10.0 and 58.9 ± 3.9 nm in diameter, respectively (mean ± SE, n=4). Size in scanning electron microscopy (SEM) was consistent with DLS measurements. Attempts to photograph liposomes by SEM were variably successful due to the liposomes' very small size (Figure 25).

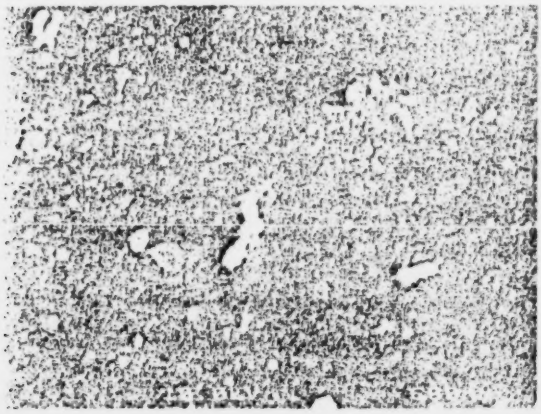


Figure 25. Scanning electron micrograph of $Soy_{PC}$ (S75) liposomes produced by high-pressure filtration. Liposomes were settled on polished carbon planchettes and fixed with 0.5% $OsO_4$ solution sputter coated with Argon to 15 nm thick and viewed using a Hitachi S-570 Scanning Electron Microscope. A distance of 600 nm is indicated by the complete distance of the series of dots in the lower right corner.

**Lipid-sperm interactions**

Having already published on the characteristics of how SL liposomes interact with porcine sperm, we focused on understanding the nature of the interaction of $Soy_{PC}$ liposomes with sperm. To monitor fusion, liposomes were made with 1 mol% R18 fluorescent fatty acid in BTS. Semen was collected from a boar and both the sperm and liposomes were analyzed separately in a flow cytometer to determine endogenous fluorescent qualities. The liposomes and sperm were then mixed and stored at 18°C under 95/5 air/$CO_2$ from 1 - 24 h, and assessed by flow cytometry at 0, 10, 30, 60 min and 24 h. Most, but not all, of the liposomes provided were absorbed by the sperm. Liposomes very quickly associated with approximately 90% of the sperm and maintained a stable association over 24 h (Figure 26).

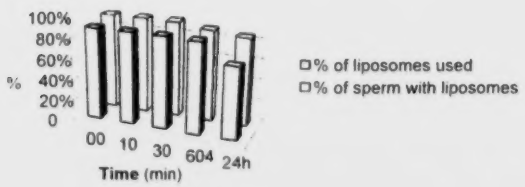



Figure 26. Soy phosphotidylcholine ($Soy_{PC}$) liposomes fused with fresh boar sperm (n=3), as measured by flow cytometry.

Fluorescence microscopy confirmed that the liposomes did not simply adhere to the sperm, but actually fused with the sperm membranes (Figure 27); simple adhesion would have been indicated by discrete vesicles attached to the sperm, whereas fusion was confirmed by the dye being uniformly spread throughout the membrane. As the flow cytometer could not distinguish between true fusion and liposomes adhering to the sperm surface, we photographed the sperm at the various incubation times (Figure 27).

In summary, $Soy_{PC}$ readily forms into liposomes of consistent small size that store well for at least 72 h, fuse to boar sperm, incorporating apparently permanently into the sperm membranes.

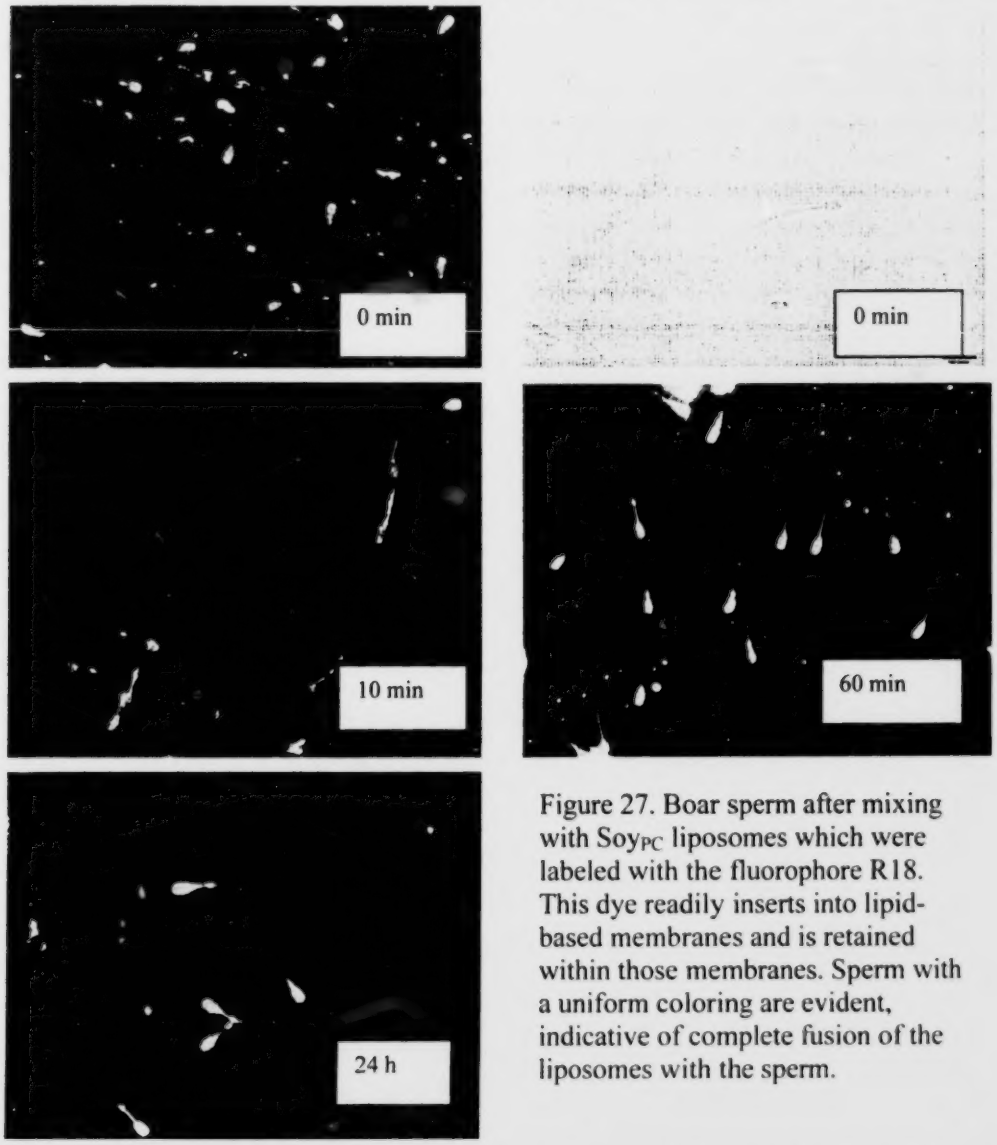



Figure 27. Boar sperm after mixing with $Soy_{PC}$ liposomes which were labeled with the fluorophore R18. This dye readily inserts into lipid-based membranes and is retained within those membranes. Sperm with a uniform coloring are evident, indicative of complete fusion of the liposomes with the sperm.

The time 0 pictures are taken of the same field as quickly as possible after mixing of liposomes and sperm. The black and white time 0 picture is a phase contrast micrograph, showing that all sperm in the field were fluorescent, supporting the data portrayed graphically in Figure 26. The 10 min picture was selected to show that small fluorescent vesicles could be identified adhering to the sperm at 0 and 10 min. These are the liposomes in their early association with the sperm. In that same photograph, other sperm with a uniform colouring were evident, indicative of complete fusion of the liposomes with the sperm. Such adherence was never seen later than 10 min after incubation. After 60 min and 24 h, sperm were uniformly fluorescent, suggesting a stable incorporation of the liposomes with the sperm.

Given the consistency of the soybean phosphatidylcholine ($Soy_{PC}$) liposome structure and the stability of the fusion, we wanted to determine if the lipids would be compatible with sperm function. Therefore, we completed a trial to assess the viability and motility of sperm exposed to one concentration of the $Soy_{PC}$ liposomes (Table 6).

Table 6. Percentage live sperm (SYBR-PI) and motile sperm (as measured by CASA) from three ejaculates (three boars) in BTS extender with either $Soy_{PC}$ liposomes or without any liposomes (Control). All values are mean ± SD.

| Time | | 0 | 15 min | 30 min | 60 min | 24 h | 48 h |
|---|---|---|---|---|---|---|---|
| % Viable | Control | | 80.0±6.7 | 60.3±9.3 | 66.7±12.9 | 62.7±2.1 | 67.0±4.2 |
| | $Soy_{PC}$ | | 78.3±13.7 | 77.3±3.5 | 71.0±8.7 | 78.3±1.2 | 76.0±5.7 |
| % Motile | Control | 55.7±23.7 | 56.7±11.4 | 53.7±13.8 | 56.0±20.1 | 56.7±27.2 | 49.3±20.0 |
| | $Soy_{PC}$ | 53.3±14.5 | 57.7±4.2 | 54.7±16.9 | 50.7±18.5 | 56.5±28.6 | 62.3±14.4 |

These results were most encouraging and we continued to do more definitive, in depth studies with various concentrations of $Soy_{PC}$ liposomes, including additional measures of sperm functionality and splitting the ejaculates so that a portion of each ejaculate was cryopreserved in addition to longer-term liquid storage.

**Effect of Soy lipids on liquid-stored boar sperm**

To be fertile, inseminated sperm must be alive, motile, able to capacitate and acrosome-react in the female reproductive tract. The storage medium must keep sperm alive, retain their ability to be motile (but not necessarily promote motility, which could use up the limited energy stores in the sperm) and prevent sperm from capacitating during storage while enabling them to undergo capacitation and the acrosome reaction when conditions resemble those in the female. Therefore, we analyzed sperm during storage in the various types of extenders for viability, for movement characteristics, and for the ability to undergo normal *in vitro* capacitation and acrosome reaction.

Liposomes were produced using the soy phosphatidylcholine lipids ($Soy_{PC}$) detailed in the previous section at 0.3, 0.5, 1.5 and 2.0 mg/mL. Select lipids modified to eliminate phosphatidylserine and increase phosphatidylcholine ($SL_{PC}$), and a 50:50 (mol:mol) mix of $Soy_{PC}$ and $SL_{PC}$ (0.3mg/mL; $Soy_{PC}$:$SL_{PC}$). Each was solubilised in chloroform:methanol (2:1, vol:vol), dried under a stream of nitrogen gas followed by 1 h in a vacuum dessicator, and then solubilised in the simple porcine extender BTS (60°C, 60 sec, vortex 30 sec; repeated three times). The lipid suspension was placed in the chamber of a syringe filter housing fitted with a 0.1 µm Nucleopore Polycarbonate Membrane (Whatman Inc. Clifton, New Jersey). The filter housing was attached to a nitrogen gas source charged to 500 psi that was opened to force the solubilised lipid through the filter.

Semen (one ejaculate from each of five Yorkshire-cross boars) was collected and sperm concentration (calibrated spectrophotometer), percentage motile (CASA) and percentage viable (SYBR/PI) sperm were determined. Semen was then diluted at 35°C to 4 x $10^7$ sperm/ml in BTS containing $Soy_{PC}$ liposomes at to final concentrations of 0, 0.3, 0.5, 1.5 and 2.0 mg/mL; or in $SL_{PC}$ (as above) at 0.3 mg/mL; or in $Soy_{PC}$:$SL_{PC}$ (1:1) at 0.3 mg/mL, and incubated for 10 min at 35°C to ensure fusion. Semen was then further diluted with BTS Thawing Solution to the final

concentration of 30 x $10^6$ sperm/mL and cooled to 25°C over 30 min. Sperm were then stored at 18°C under 5% $CO_2$ and tested for viability, motility, and acrosomal status (FITC-PNA + PI staining after 0 and 6 h of incubation in capacitation medium or BTS, followed by 30 min ± the calcium ionophore A23187). The remainder of the semen was stored at 18°C and function tested after 24, 48, 72 and 168 h (7 days) of storage.

Porcine sperm stored in the higher concentrations of soy liposomes ($Soy_{PC}$) appeared to have higher percentages of viable sperm over time of liquid storage; viability and motility was extended over 7 days (Figure 28). Such preparations could be of great assistance for pig producers in maintaining sperm function for artificial insemination over a reasonable period of time. These data suggest that addition of the lipids can enhance the survival rate of boar semen for artificial insemination.

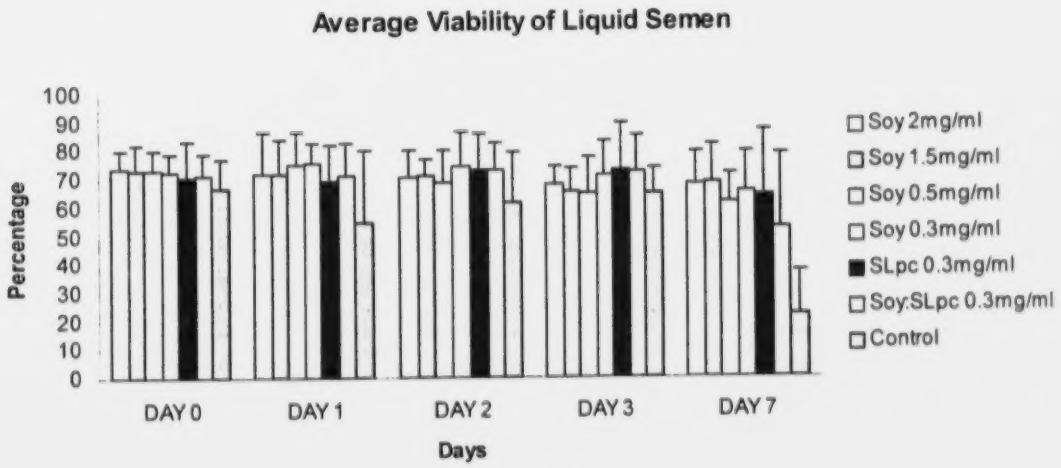



Figure 28. The percentage of viable boar sperm (SYBR-PI) in various lipid treatments in BTS; control sperm were in BTS alone. The average overall viability was higher with lipids than without, and after 7 days storage, significantly more sperm were alive in BTS with liposomes, than BTS alone ($P<0.05$). Each bar represents the mean of five different ejaculates ± SD.

The total percentage of motile sperm was, as expected, lower than the percentage viable, and lower than would normally be expected (Figure 29). The percentage of sperm that were motile was greater for sperm in BTS (control) than those in BTS with 2 mg/mL $Soy_{PC}$ liposomes after 2 days of storage, but there was no significant difference on any other day. In addition, CASA parameters focused on velocity (particularly average path and curvilinear velocity) and amplitude of lateral head displacement (an indicator of hyperactivation).

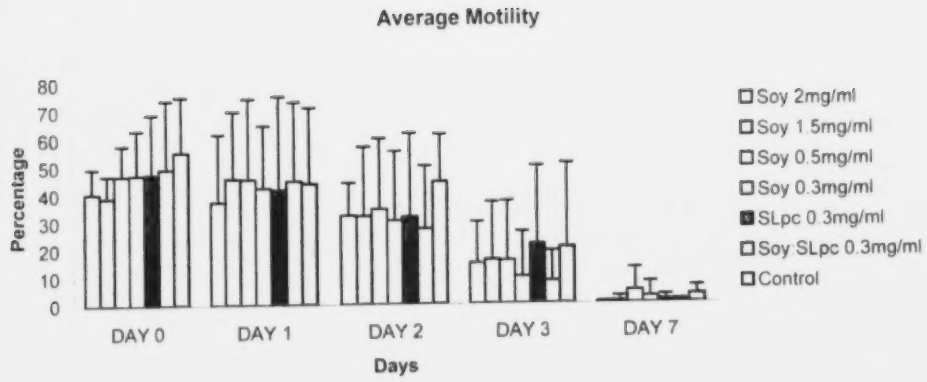


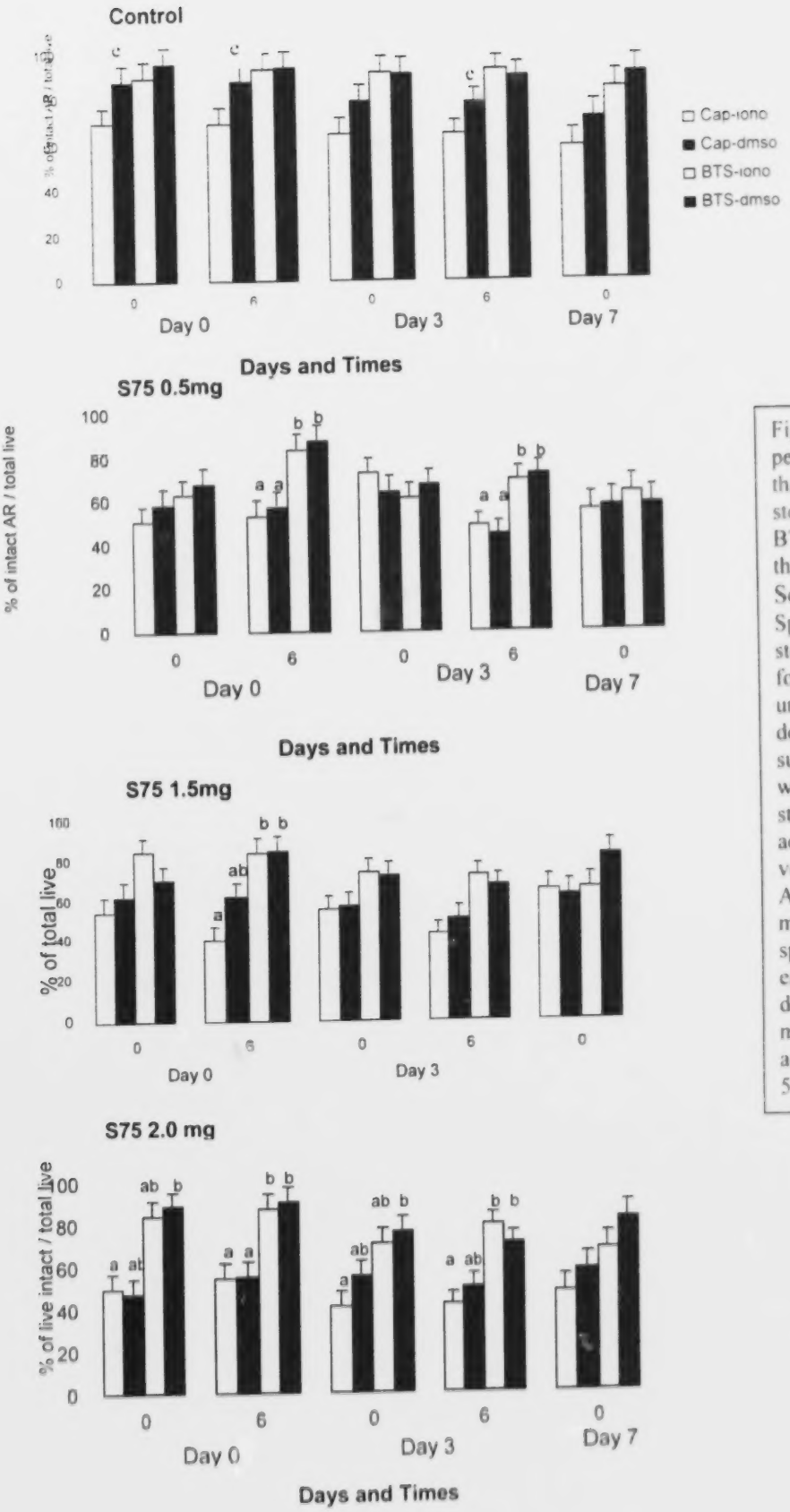

Figure 29. The percentage of motile boar sperm (as determined by CASA) after storage at 18°C in various lipid treatments in BTS; control sperm were in BTS alone. The percentage of sperm that were motile was greater for sperm in BTS (control) than those in BTS with 2 mg/mL $Soy_{PC}$ liposomes after 2 days of storage ($P<0.05$), but there was no significant difference on any other day. Values are mean ± SD of five ejaculates.

Capacitation status was measured in sperm taken from the extenders and put into either plain BTS or 'capacitation medium' for either 0 or 6 h, which should help the sperm capacitate. At each of those times, some sperm were given an 'ionophore', which should make capacitated sperm undergo the acrosome reaction. Ideally, the sperm at 0 h should not acrosome-react, and after 6 h they should. Using a flow cytometer, we classified thousands of sperm from each time and treatment as live or dead, and then determined the acrosomal status of the live sperm as acrosome-reacted, partially reacted, or intact, and then expressed these as percentages. Results are presented for the live intact sperm, which is the inverse of the (partially- plus completely-reacted-) cells.

All the treatments on Day 0 seem to follow a 'normal' trend for capacitation medium and the ionophore-supported acrosome reactions (Figures 30 - 33 selected treatments). After 3 days of storage, there was a loss of the ionophore effect on all sperm except those stored in $Soy_{PC}$ at 2 mg/mL and 1.5 mg/mL. On Day 7, there was a loss of the ionophore effect as well as a loss of the control medium effect. Only sperm stored in $Soy_{PC}$ at 2 mg/mL and $SL_{PC}$ at 0.3 mg/mL (data not shown) followed a 'normal' acrosome assay trend on Day 7.

The percentage of live sperm that were acrosome-intact did not differ among days in the control sperm within capacitation media. After the first 3 days of storage in 0.5 mg/mL $Soy_{PC}$, and after 7 days in 0.3 mg/ml $Soy_{PC}$, acrosome reactions were adversely affected.

These analyses examined the acrosomal status of the live cells in the population. Data were also analyzed to express the intact and acrosome-reacted live sperm as a percentage of all sperm present, alive and dead. After 7 days in BTS alone, many fewer sperm were alive and capable of normal acrosome reactions, while sperm stored in $SL_{PC}$ at 0.3 mg/mL were variably able to acrosome-react normally depending on the day of storage. There were no other significant differences between live cell and total cells for both intact and reacted sperm.




Figures 30 - 33. The percentage of live boar sperm that are acrosome-intact after storage for 0, 3 or 7 days in BTS alone or BTS containing the indicated concentration of $Soy_{PC}$ (S75) liposomes. Sperm were taken from storage (0 h) and incubated for 6 h in a 37°C water bath under 5% $CO_2$ in media that does (CAP) or does not (BTS) support capacitation. Sperm were then exposed to a stimulator (iono) of the acrosome reaction or its vehicle control (DMSO). Acrosomal status was measured in 2000-10000 sperm by flow cytometer at each time. The numbers displayed are least-squares means ± SE of intact (non-acrosome-reacted) sperm, n = 5 boars.

Because the $Soy_{PC}$ liposomes provided some enhanced longevity to sperm, but there was still room for improvement, particularly with cryopreservation, liposomes of $Soy_{PE}$ were evaluated. DLS assessed their size to be relatively similar to those of $Soy_{PC}$ and they fused rapidly, with some slight reversibility, to boar sperm. Sperm were viable and motile after 24 h, therefore we undertook a full 10 day storage trial with $Soy_{PE}$ liposomes at 2, 1.5, 0.5 and 0.3 mg/mL, plus $Soy_{PC}$:$Soy_{PE}$ (1:1, wt:wt) at 1.5 and 0.5 mg/mL (total). Four ejaculates of different boars were extended and split into identical aliquots and immediately mixed with one of seven treatments and stored at 18°C, testing motility and viability on Days 0, 1, 2, 3, 5, 7, 8, 9 and 10 and acrosomal integrity on Days 0, 3, 7 and 10. Sperm function was assessed by CASA, viability with SYBR-14/PI, and acrosomal integrity at 0 and 5 h of incubation.

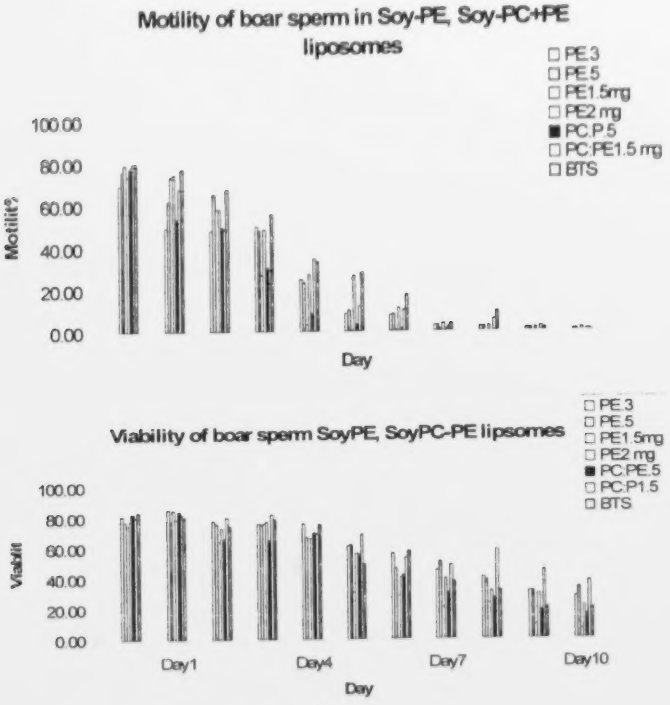



Figure 34. Five boar ejaculates were split and extended in BTS with the indicated amount of Soy liposomes. Viability and motility were assessed by CASA and SYBR-PI. The 0.5 mg $Soy_{PE}$ and 1.5 mg $Soy_{PC}$-$Soy_{PE}$ mix was more protective of sperm ($P<0.05$).

Despite the positive results with of the $Soy_{PE}$ liposomes on sperm motility and viability, these liposomes did not support good acrosomal structure and function. Exposing boar sperm to BTS containing 1.5 mg/ml $Soy_{PC}$/$Soy_{PE}$ liposomes caused an immediate decline in the percentage of live, acrosome-intact cells compared to sperm in BTS alone or BTS containing $Soy_{PE}$ liposomes. This reduction was still evident after 3 or 7 days of storage, although by 10 days all three treatments had similar low percentages of live acrosome-intact sperm.

It is important for any extender to prevent premature capacitation of the sperm, which is measurable at 0 h incubation on the various days of storage. Exposure to $Soy_{PC}$/$Soy_{PE}$ liposomes immediately induced a significant loss of acrosomes in live sperm; $Soy_{PE}$ alone caused a numerical, but non-significant, loss. Storage in liposomes resulted in a numerical, but non-significant, reduction in live intact sperm at each of the subsequent storage days.

It is also important for any extender to permit sperm to acrosome-react in response to capacitating conditions; a relatively similar percentage of sperm from each extender underwent the acrosome reaction after incubation in the capacitating medium for 4 h. In addition, we found that acquisition of $Soy_{PE}$ was difficult and expensive, and so the use of this lipid was discontinued.

**Effect of Soy lipids on cryopreserved boar sperm**

Semen from the same ejaculates as for the liquid semen trial was used, and frozen by our standard protocol modified slightly to accommodate the liposome treatment. Semen was initially diluted 1:1 with the treatments, cooled to 25°C over 2 h, centrifuged (800 x g, 10 min) and resuspended on BF5-A containing the liposomes at double the final desired concentration. Control semen was diluted in BF5A containing egg yolk. Semen was cooled to 5°C over 4 h, diluted in BF5-B containing glycerol (final concentration 3%), packaged in 0.5 mL straws, cooled in liquid nitrogen vapors, and plunged into liquid nitrogen. Normally, we would cool and freeze in controlled-rate cooler, but our unit was not functional, so rather than stop all work, we utilized the tried-and-true (but tedious) non-mechanical method. After a minimum of 72 h storage, straws were thawed in 60°C water bath for 5 sec, diluted in BTS, and motility and viability tested. The percentage of sperm that were viable was determined by SYBR-PI staining and the percentage of motile sperm was determined by CASA (≥200 sperm).

Pre-freeze sperm viability was 70.5 ± 19.2%. Unfortunately, all sperm in the lipid treatments were dead and non-motile; control sperm appeared reasonably functional, indicating that the cryopreservation protocol itself was not at fault (Table 7). This clearly indicates that the liposomes at the tested concentrations harm sperm subjected to the freezing and/or thawing processes.

Table 7. Post-thaw motility and viability of boar sperm after cryopreserving identical aliquots from each ejaculate in BF5 containing the indicated treatments

| | $Soy_{PC}$ 2mg/mL | $Soy_{PC}$ 1.5mg/mL | $Soy_{PC}$ 0.5mg/mL | $Soy_{PC}$ 0.3mg/mL | $SL_{PC}$ 0.3mg/mL | $Soy_{PC}$:$SL_{PC}$ 0.3mg/mL | Control |
|---|---|---|---|---|---|---|---|
| % Viable | 0 | 0 | 0 | 0 | 0 | 0 | 19.2 ± 3 |
| % Motile | 0 | 0 | 0 | 0 | 0 | 0 | 41.5 ± 5 |

**Field breeding trial with liquid extended semen**

We are now completing a field trial with the $Soy_{PC}$ 1.5 mg/mL in a commercial swine operation. The producer was selected in consultation with Dr R. Friendship, as directed by the Scientific Committee. The trial had to be designed to acquire his participation, so sows were inseminated with the $Soy_{PC}$ extender on Day 0 or 7 of storage (n=25 for each day), and compared to control sows who were inseminated with fresh semen in BTS extender on those same days of storage (n=25 for each day). Semen was collected from the producer's own boars, selected on the basis of his breeding program. There have been a number of problems associated with farm staff being unfamiliar with the need to keep treated semen for experimental use, to wean sows at pre-determined times and to conduct pregnancy tests on time. Success has thus far been determined

by a 21 day non-return rate. Thus far, the overall pregnancy rate for control sows (n=30) is 73% (range 60-100; note that this is a normal average for this farm); sows inseminated with $Soy_{PC}$ on Day 0 of storage had an average pregnancy rate of 65% (range 0-100); and sows inseminated with $Soy_{PC}$ after 7-9 days of storage was 54% (range 20-100%; the lowest rate was with a Day 9 insemination). The remaining sows will be inseminated or pregnancy-checked the first week of March, and final results will be prepared as soon as possible, but the final insemination groups will not be pregnancy-checked until March 22.

## CONCLUSIONS AND RECOMMENDATIONS

Overall, this project has taken longer than it should have to accomplish our stated objectives, primarily because of the different institutions involved, and the fact that the Principal Investigator left the University of Saskatchewan, and then Canada. The delay in negotiating and finalizing the research agreement set the project back more than a year and a half. However, research milestones and objectives were finally accomplished and important results have been generated. For each species, the plant-based phospholipid extender that was most efficacious was characterized as to efficacy in preserving spermatozoa during cryopreservation and/or prolonged exposure.

For the bovine synthetic extender, the phospholipid preparation ($Soy_{PC}$ plus canola oil; 1-1.5) was most efficacious and is expected to be an effective extender for the cryopreservation of bull semen. The industry partners are very excited about its potential for commercialization, and are in discussions as to how this might be accomplished. Although field trials are not yet complete, data retrieved so far, show no difference in pregnancy rate when compared to a very efficacious commercial egg yolk extender. In addition, fertilization rates in superstimulated donor cows did not differ from that achieved with semen from the same bulls cryopreserved in the egg yolk extender. Not only is the synthetic extender highly efficacious, but it is also free of animal products and has a shelf-life of more than 8 months. On the other hand, if regulatory authorities require that autoclaving of the extender for export purposes, efficacy was retained for up to 3 months after autoclaving. No other extender available today can make that claim.

The porcine synthetic extender was as expected somewhat more difficult to develop. The phospholipid preparation that was most efficacious was 1.5 mg/mL $Soy_{PC}$ (without canola oil), however, a preparation in canola oil has not been thoroughly evaluated. The addition of 1.5 mg/mL $Soy_{PC}$ to the Beltsville (BTS) extender would appear to preserve porcine semen for up to 7 days. However, field data are still being retrieved. Cryopreservation of porcine semen with the synthetic extender was unsuccessful. It is apparent that a great deal more study will be required. However, the observation that $Soy_{PC}$ readily forms into liposomes of consistent small size that fuse and are incorporated into boar sperm membranes, apparently permanently, offers great hope that we are on the correct path. The collaborating partners have agreed that once all field data are compiled and statistical analysis is completed, they will again meet and make plans for the next steps, which will undoubtedly mean more experimentation.

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